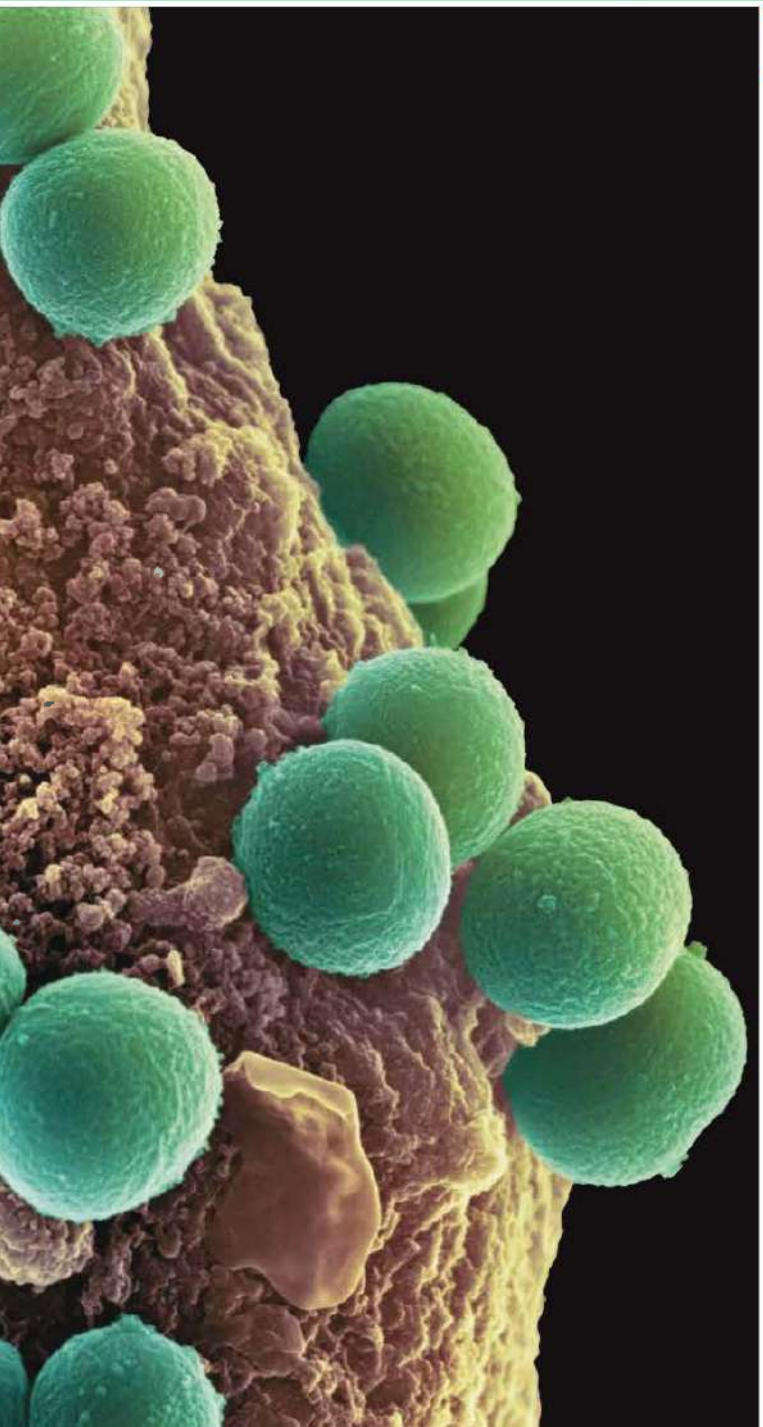
MICROBIOLOGY
Laboratory Theory & Application
FOURTH EDITION
Michael J. Leboffe
Burton E. Pierce

MICROBIOLOGY



Laboratory Theory & Application

FOURTH EDITION

Michael J. Leboffe
San Diego City College

Burton E. Pierce

MORTON
PUBLISHING
925 W. Kenyon Avenue, Unit 12
Englewood, CO 80110
www.morton-pub.com

Book Team

Publisher	Douglas N. Morton
President	David M. Ferguson
Acquisitions Editor	Marta R. Martins
Project Editor	Rayna S. Bailey
Editorial Assistant	Sarah D. Thomas
Production Manager	Joanne Saliger
Production Assistant	Will Kelley
Illustrations	Imagineering Media Services, Inc.

Copyright © 2015 by Morton Publishing Company

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording, or otherwise, without the prior written permission of the publisher.

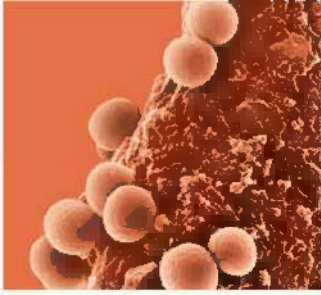
Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

ISBN-10: 1-61731-250-9
ISBN-13: 978-1-61731-250-2

Library of Congress Control Number: 2014947059

PREFACE



Back in college I remember balking at writing a term paper, or even an essay. I never dreamed I'd be writing the preface to a 900+ page, college-level, 4th edition microbiology laboratory manual. I imagine Dr. Kelly, my General Microbiology professor at San Diego State University, would have felt the same way had she given it any thought. I also figured that hers would be the last microbiology course I'd ever take or need. (I can state with pride, however, I *did* earn a B in that course.) Silly me, making plans.

Nevertheless, odds and odd career paths be damned: here I am. America, Land of Opportunity, indeed!

This was supposed to be a "simple" revision. After all, the thinking went at Morton Publishing, with three previous editions of this book (hereafter referred to as *MLTA* 4e) and two editions of its spawn, *Microbiology Laboratory Theory and Application, Brief Edition* (*MLTAB* 2e), a new edition of this book would only involve a little touching-up here, a little trimming there, and addressing reviewer suggestions. It sounded like a delightful plan and I embraced it at first. But, it didn't play out that way. For some reason, I don't (won't? can't?) work that way. When I see things that I think can be improved, I want to improve them. In my book (literally and figuratively), a revision is a *revision*, not a tune-up. So, some significant changes have been made and these are outlined below.

The other reason this revision was more challenging than originally planned is that my longtime friend and coauthor, Burt Pierce, retired. (For the second time! He took a pass on revising *A Photographic Atlas for the Microbiology Laboratory*, 4e, but rejoined me for *MLTAB*, 2e. After the wonderful retirement party Morton Publishing threw for him, I think it will "stick" this time. Still, the door is open for a return as far as I'm concerned. Burt? BURT?) As a consequence, the book became my sole responsibility and turned into an undertaking bigger than I anticipated. Lesson learned: There's a reason most of your textbooks have multiple authors.

Lastly, please notice in the descriptions of revisions, deletions, and additions that follow, I use the first person plural pronoun–we–because Burt's influence is still all over this book. Even though the discussions regarding what changes should be made were one-sided in my head, his commitment to quality and not underestimating our audience's abilities continued to influence me.

A couple of global changes addressing safety in the microbiology laboratory were made throughout the book. From the first edition, we have always emphasized using techniques that minimize aerosol production, but this edition places an even greater emphasis on good technique, with reminders throughout the text. The other major change has been a reduction in the number of BSL-2 organisms used. Where possible, they have been replaced with suitable substitutes that give the same result. In other cases, they have been listed as "optional." In still others where no replacements are available, they remain. Please pay attention to the organisms you are working with and use appropriate caution. As always, it is ultimately up to the instructor to use his or her professional judgment and choose organisms that are suitable for the course level and lab facilities of their institution and to each student to follow standard safety guidelines.

Another global change is that the data sheets have been moved from the back of the book to immediately following the corresponding exercise. Even though the book is loose leaf and pages can be moved easily, having them with the exercise was a common suggestion made by reviewers. And now they are. Attention was also paid to the questions, and in many exercises the questions have been re-sequenced, reworded, split into parts, or entirely replaced. Because there are more exercises than can be done in any one-semester course, there is also repetition

of certain questions from exercise to exercise because they address particularly important points and we wanted to ensure that students have to answer them regardless of which exercises are chosen.

One feature of *MLTA* that seems to please adopters of previous editions has been the visual content—photos and art. In this edition, over 90 photographs have been replaced and 20+ new photos have been added. In addition, there are 20+ new pieces of artwork and more than 35 pieces have been revised or replaced.

Virtually all lab exercises were rewritten to a greater or lesser degree in an attempt to bring greater clarity to the theory, application, and instructions for each. Following are some highlights of specific topics added or revised in each section.

- **Introduction—Safety and Laboratory Guidelines** A section devoted exclusively to handling BSL-2 organisms has been added, as was a sample "safety contract" to be signed by the student agreeing to comply with safety regulations outlined in this book and as amended by their institution. The topic of controls was also revised.
- **Section 1—Fundamental Skills for the Microbiology Laboratory** A hand washing exercise (Exercise 1-1) comparing different cleansing agents replaces the Glo Germ™ exercise from the previous edition. (But it is still available through Morton's Custom Publishing, if desired.) Exercise 1-3 (Common Aseptic Transfers and Inoculation Methods) has been modified and presents transfers using a different approach. Admittedly, previous attempts at describing these were cumbersome and could be hard to follow. We think this is an improvement (and it has been test driven in *MLTAB*, 2e). Exercise 1-4 (Streak Plate) has new photos and the T-streak inoculation has been added as an option to the quadrant streak. Exercise 1-5 (Spread Plate) has been modified by advising the use of a screw-cap jar to hold the alcohol for flaming the glass rod in case the alcohol catches fire.
- **Section 2—Microbial Growth** While the basic protocols for each exercise in this section are unchanged, each has been rewritten for greater clarity and integration.
- **Section 3—Microscopy and Staining** Exercise 3-1 (Introduction to the Light Microscope) now includes instructions on using Köhler illumination in addition to enhanced instructions on general microscope use. Exercise 3-3 (Microscopic Examination of Eukaryotic Microbes) and Exercise 3-4 (Microscopic Examination of Freshwater Microbes) were totally reorganized to reflect a more current taxonomy of eukaryotes. Minor revisions were made to the bacterial stain exercises for clarity and safety. The Parasporal Crystal stain was removed.
- **Section 4—Selective Media** Most photos in this section were replaced to show spot inoculation rather than some other pattern (which might have been more photogenic, but was confusing because the instructions say to spot inoculate).
- **Section 5—Differential Tests** This section was the recipient of many replacement photos. It also was treated to a sequence change. ONPG (Exercise 5-5) is now in the fermentation group of exercises, and the exercises involving hydrolytic enzymes have been reorganized to group together similar substrates.
- **Section 6—Quantitative Techniques** Exercise 6-1 (Standard Plate Count) discusses other methods of counting colonies and explains the rationale for using "Original Sample Volume" rather than "Plate Dilution Factor" in calculations. Exercise 6-3 (Direct Count) explains the Petroff-Hausser counting chamber construction more fully and the artwork was revised to show cells on the grid and how to handle cells landing on the lines. The Closed-System Growth exercise (6-4) now includes additional suggested incubation temperatures, which can be used or not depending on an institution's facilities and budget. Finally, the instructions for Exercise 6-6 (Thermal Death Time versus Decimal Reduction Value) have been rewritten.

- **Section 7—Medical Microbiology** The Theory sections of Exercises 7-1 (Snyder Test) and 7-2 (Lysozyme Assay) were rewritten. Two optional antibiotics have been added to the materials list for the Kirby-Bauer test (Exercise 7-3) and the Theory was rewritten with more detail about the test and its history. Exercise 7-5 (MMWR Report) was updated to reflect changes in the CDC website and changes to the list of notifiable diseases. The instructions for Exercise 7-6 (Epidemic Simulation) were rewritten to emphasize the safe execution of the lab. Lastly, the identification flow charts in Exercises 7-7, 7-8, and 7-9 were revised to remove most BSL-2 organisms and minor changes were made to the tests used.
- **Section 8—Environmental Microbiology** This section had the least done to it. The labs are basically the same, but some errors were corrected, a few photos were replaced with better ones, and the introductory material and the box discussing trophic groups were rewritten. Most exercises had changes intended to improve clarity and materials lists were made more comprehensive. Exercise 8-13 (Multiple Tube Fermentation Method for Determining Water Quality) received the greatest attention and was rewritten.
- **Section 9—Food Microbiology** This is the shortest section, with only three lab exercises but it has grown the most—by 50%, because one lab is brand new: Yogurt CFU Density of an Active and Live Culture and Identification of a Yogurt Unknown (Exercise 9-3). It could have gone in Section 6, but because the plate counts are used to check numbers of microbes in yogurt products with the label "Live & Active Cultures," we thought it fit better in this section. After determining cell density in a yogurt sample, students have the opportunity to identify isolates.
- **Section 10—Microbial Genetics** This section has two, totally rewritten exercises supplied by Amy Warenda Czura. They are Exercise 10-2 (Restriction Digest) and Exercise 10-4 (Polymerase Chain Reaction). In previous editions these utilized commercial kits and now they are "made from scratch" protocols. These are accompanied by extensive new artwork, as is Exercise 10-3 (Bacterial Transformation). There are even photos of the jellyfish species that naturally produces green fluorescent protein!
- **Section 11—Hematology and Serology** Antigen and antibody structure and function are now described more fully in a separate "box" preceding the serology exercises (Exercises 11-2 through 11-5), Exercise 11-5 (Blood Typing) now explains more thoroughly the genetic and molecular basis for blood types in the ABO system, and a new ELISA exercise (Exercise 11-6) using a kit from BioRad Laboratories has replaced the previous one. The Mononucleosis Hemagglutinin Test has been removed.
- **Section 12—Eukaryotic Microbes** This entire section was virtually rewritten to reflect current taxonomy and to enhance and update descriptions of diseases.
- **Appendices** The biochemical pathways in Appendix A have been modified to show additional intersecting pathways and have been redrawn to more clearly illustrate the intermediates in the primary path versus those on intersecting paths. Appendices illustrating transfers (B, C, and D) have been rewritten to include BSL-2 precautions. Appendix F (Alternative Procedures) has also been rewritten and the procedural diagrams have been redrawn for greater clarity. Appendix G (Electrophoresis) was rewritten and new artwork was added. Finally, the media and solutions recipes in Appendix H were updated.

Those are the highlights. My reaction? I think of the The Grateful Dead's lyrics: What a long, strange trip it's been. Amen, Jerry and the Band!

All the best,

Mike
La Mesa, CA
December 2014

Acknowledgments

Many years ago, a San Diego City College president addressed the faculty and staff and used the saying, "None of us is as good as all of us," as a motto for getting the college to pull together and "do more with less." It was true in that context and it is equally true in the production of a textbook. The author has his/her name on the cover, but publishing is so complex these days that it takes dozens of people to produce even a modest effort like this book. Most operate in the background, but their contributions are essential. The challenge facing me now is that after almost 20 years of publishing with Morton Publishing Company (I've hung on long enough to see three different Morton logos on my books), it is a bit of a challenge to find new, creative ways to thank these people who have played such an enormous part in making my forays into authorship more successful than I probably deserve. Nevertheless, acknowledgement of gratitude never gets old and is never out of place, especially when it is so richly deserved.

Morton Publishing was founded by Doug Morton, who had a vision of producing high-quality textbooks at a reasonable price, a business model that has been wildly successful for his company and is still its guiding philosophy. Doug has transferred the day-to-day operation of his company into the hands of three young, highly capable individuals: President David Ferguson, Vice President of Operations Chrissy DeMier, and Vice President of Sales and Marketing Carter Fenton. These people have been charged with the task of navigating Morton Publishing through the challenging publishing landscape of the early 21st century, and I feel very secure with them at the helm. I thank them for their support, encouragement, patience (with a capital "P"!), and friendship during my collaboration with Morton Publishing.

Coinciding with its success, Morton Publishing has grown in size and now houses an Editorial Department! Thanks to Marta Martins, Senior Acquisitions Editor, who administered peer reviews of the third edition and collated the responses into a manageable form that I could respond to easily. Thanks also to Editorial Assistant Sarah Thomas for her role in proofreading and performing miscellaneous tasks. As a book nears completion at Morton, the boundaries of the Editorial Department become blurred and folks with other titles take on the additional burden of proofreading (which to me is akin to having one's fingernails pulled out with pliers). Some of these folks have already been mentioned; one who was not is Jessica Smith, Marketing Manager, who executes the marketing strategy and proofreads in her "spare" time. Thanks, Jes. And remember: head up, elbows down.

Special thanks go to Rayna Bailey, Project Editor. Rayna edited the manuscript, obtained permissions, coordinated the art program, and communicated with virtually everyone associated with this book so I could concentrate on writing. Her positive attitude and gentle prodding (but only when absolutely necessary) kept me on task and moving forward, even as I was pathetically behind schedule. I say without qualification that I couldn't have done this without her. Thank you, Rayna. You rock!

The production team at Morton has once again done a masterful job in designing this book with their customary artistic flair. Art meets science at Morton Publishing! Thanks to Joanne Saliger, Production Manager, and Will Kelley, Production Assistant, for applying their talents to my book and for their patience with the pace at which the manuscript was received. Another job well done.

Thanks to Scott Day, Sales Manager, and the all the sales representatives who meet potential adopters and present my books in the best light possible. Without them, my closets would be full of unsold books! Their job is a difficult one and I appreciate their efforts more than they can know.

I extend my heart-felt gratitude to everyone who works in the Morton office for embracing me as one of their own during my occupancy of office space in August 2014. You made a challenging situation for me a pleasant one. Visit www.Morton-pub.com and meet all of these people!

Thanks are also due my colleagues at San Diego City College—past and present. Current faculty Jake Brashears, Jennifer Chambers, Roya Lahijani, Erin Rempala, Dave Singer, and Gary Wisehart tolerated my pinball-like presence in the hallways as I raced from classes, office hours, and meetings between writing obligations. "Hello!" became an in-depth conversation over the last year. So now, let's talk sometime. Soon. City College microbiology instructors Tom Kaido, Sabine Kurz-Camcho, Martha Myers, and Brett Pickett used the book in their classes, offered suggestions, and provided me with a sounding board for new ideas, as did my San Diego State University teaching interns Heather Heinz and Polly Parks. Dean (and former office partner) of the School of Engineering & Technologies, Mathematics, Sciences, and Nursing, Minou Spradley, oversaw the process of my renting San Diego City College lab facilities. (These things go so smoothly when I let someone else handle them!) Last but not least, biology lab technicians Deb Reed, Laura Steininger, and Muu Vu were always ready to cheerfully assist me in finding materials I needed that I should have learned the location of years ago. Thank you, one and all.

I engaged in many conversations about safety in the microbiology teaching laboratory with colleagues Marlene DeMers and Tom Gibson, both of San Diego State University. They had a profound influence on my presentation of that topic in this fourth edition. Thanks, you two. Enjoy your retirements!

I also want to recognize the contributions of hand models Diana Carrillo, Heather Heinz, Anita Hettena, Nathan Leboffe, Deb Reed, Burt Pierce, Carla Sweet, and Gary Wisehart. I know which hands are yours and I get a warm, fuzzy feeling remembering each photograph and knowing which hands belong to whom even if the majority of readers probably don't notice the differences. Oh, and just to show that I'm keeping up with the times, there are also a couple of "selfies" in the book. See if you can find them…

Thanks to the reviewers of the third edition who provided me with comprehensive feedback and many useful suggestions. These are: Patricia Clinard Alfing, Davidson County Community College; Richard Adler, University of Michigan–Dearborn; Amy Warenda Czura, Suffolk County Community College–Eastern Campus; Timothy Ladd, Millersville University; and Jeanette M. Loutsch, University of Science and Arts of Oklahoma.

In addition to providing a review, Amy Warenda Czura jumped in at the 11th hour and developed protocols for a restriction digest lab (Exercise 10-2) and a PCR lab (Exercise 10-4), as well as a new protocol for electrophoresis (Appendix G). Not only did she get a late call, she had the equally challenging task of writing new protocols that fit the framework and style of the existing labs she was replacing. You were a lifesaver, Amy.

Burt Pierce, my former coauthor, also deserves recognition because without his contributions there wouldn't have been a second edition, much less a fourth. His contributions and influence are still vital parts of this book.

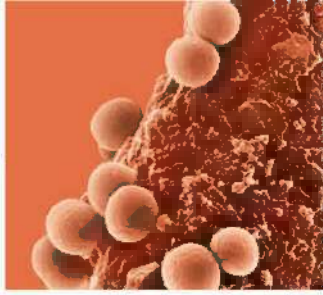
To all the others: There have been so many who have contributed in various ways over four editions of *MLTA*, two editions of *MLTA Brief*, and four editions of the *Photographic Atlas for the Microbiology Laboratory* that it has become impractical to identify everyone individually, but your collective efforts have not been forgotten and continue to be appreciated. One big, hearty "Thanks" to you all.

None of us is as good as all of us. Indeed.

Dedication

I dedicate this book to David Singer and Gary Wisehart, two long-time friends and colleagues, who, more than anyone else, have influenced my development into the educator that I am. Amazingly, they have accomplished this more than they probably realize. Thank you. Gentlemen, this book is done. When does Padres season start?

CONTENTS



INTRODUCTION **Safety and Laboratory Guidelines** 1

Student Conduct 3
Basic Laboratory Safety 4
Reducing Contamination of Self, Others, Cultures, and the Environment 5
Guidelines Governing Handling of BSL-2 Organisms 5
Disposing of Contaminated Materials 6
Student Compliance with Laboratory Safety Regulations 7
A Word About Experimental Design 8
Data Presentation: Tables and Graphs 9
Data Presentation: Be Creative, But Complete 10
Student Safety Contract 13

SECTION 1 **Fundamental Skills for the Microbiology Laboratory** 15

EXERCISE 1-1 A Comparison of Hand-Cleansing Agents 17
♦ **Basic Growth Media** 21
EXERCISE 1-2 Nutrient Broth and Nutrient Agar Preparation 21
♦ **Aseptic Transfers and Inoculation Methods** 27
EXERCISE 1-3 Common Aseptic Transfers and Inoculation Methods 29
EXERCISE 1-4 Streak Plate Methods of Isolation 41
EXERCISE 1-5 Spread Plate Method of Isolation 49

SECTION 2 **Microbial Growth** 55

♦ **Diversity and Ubiquity of Microorganisms** 57
EXERCISE 2-1 Ubiquity of Microorganisms 57
EXERCISE 2-2 Colony Morphology 63
EXERCISE 2-3 Growth Patterns on Slants 75
EXERCISE 2-4 Growth Patterns in Broth 79
♦ **Environmental Factors Affecting Microbial Growth** 83
EXERCISE 2-5 Evaluation of Media 83

♦ **Aerotolerance** 91
EXERCISE **2-6** Agar Deep Stabs 91
EXERCISE **2-7** Fluid Thioglycollate Broth 95
EXERCISE **2-8** Anaerobic Jar 99
♦ **Effect of Environmental Factors on Bacterial Growth Rate** 103
EXERCISE **2-9** The Effect of Temperature on Microbial Growth 103
EXERCISE **2-10** The Effect of pH on Microbial Growth 109
EXERCISE **2-11** The Effect of Osmotic Pressure on Microbial Growth 113
♦ **Control of Pathogens: Physical and Chemical Methods** 121
EXERCISE **2-12** Steam Sterilization 123
EXERCISE **2-13** The Lethal Effect of Ultraviolet Radiation on Microbial Growth 129
EXERCISE **2-14** Chemical Germicides: Disinfectants and Antiseptics 135

SECTION **3** **Microscopy and Staining** **141**
EXERCISE **3-1** Introduction to the Light Microscope 143
♦ **Köhler Illumination** 147
EXERCISE **3-2** Calibration of the Ocular Micrometer 155
EXERCISE **3-3** Microscopic Examination of Eukaryotic Microbes 161
EXERCISE **3-4** Microscopic Examination of Freshwater Microbes 169
♦ **Bacterial Structure and Simple Stains** 181
EXERCISE **3-5** Simple Stains 185
EXERCISE **3-6** Negative Stains 191
♦ **Differential and Structural Stains** 195
EXERCISE **3-7** Gram Stain 195
EXERCISE **3-8** Acid-Fast Stains 203
EXERCISE **3-9** Capsule Stain 211
EXERCISE **3-10** Endospore Stain 215
EXERCISE **3-11** Wet Mount and Hanging Drop Preparations 221
EXERCISE **3-12** Flagella Stain 225
EXERCISE **3-13** Morphological Unknown 229

SECTION 4 **Selective Media** 235

♦ **A Word About Selective Media** 236

♦ **Selective Media for Isolation of Gram-Positive Cocci** 237

EXERCISE 4-1 Phenylethyl Alcohol Agar 237

EXERCISE 4-2 Columbia CNA With 5% Sheep Blood Agar 243

EXERCISE 4-3 Bile Esculin Agar 249

EXERCISE 4-4 Mannitol Salt Agar 253

♦ **Selective Media for Isolation of Gram-Negative Rods** 259

EXERCISE 4-5 MacConkey Agar 259

EXERCISE 4-6 Eosin Methylene Blue Agar (Levine) 267

EXERCISE 4-7 Hektoen Enteric Agar 273

EXERCISE 4-8 Xylose Lysine Desoxycholate Agar 279

SECTION 5 **Differential Tests** 285

♦ **A Word About Biochemical Tests and Acid-Base Reactions** 286

♦ **Introduction to Energy Metabolism Tests** 287

EXERCISE 5-1 Reduction Potential 289

EXERCISE 5-2 Oxidation–Fermentation Test 295

♦ **Fermentation Tests** 303

EXERCISE 5-3 Phenol Red Broth 303

EXERCISE 5-4 Methyl Red and Voges-Proskauer Tests 311

EXERCISE 5-5 ONPG Test 317

EXERCISE 5-6 Catalase Test 321

EXERCISE 5-7 Oxidase Test 327

EXERCISE 5-8 Nitrate Reduction Test 333

♦ **Nutrient Utilization Tests** 339

EXERCISE 5-9 Citrate Utilization Test 339

EXERCISE 5-10 Malonate Utilization Test 345

♦ **Decarboxylation and Deamination Tests** 349

EXERCISE 5-11 Decarboxylation Tests 349

EXERCISE 5-12 Phenylalanine Deaminase Test 357

♦ **Tests Detecting Hydrolytic Enzymes** 361
EXERCISE **5-13** Starch Hydrolysis (Amylase Test) 361
EXERCISE **5-14** DNA Hydrolysis (DNase Test) 367
EXERCISE **5-15** Lipid Hydrolysis (Lipase Test) 371
EXERCISE **5-16** Casein Hydrolysis (Casease Test) 375
EXERCISE **5-17** Gelatin Hydrolysis (Gelatinase Test) 379
EXERCISE **5-18** Urea Hydrolysis (Urease Test) 383
EXERCISE **5-19** PYR Test 389
♦ **Combination Differential Media** 393
EXERCISE **5-20** SIM Medium
Sulfur Reduction, Indole Production, Motility 393
EXERCISE **5-21** Triple Sugar Iron Agar / Kligler Iron Agar 401
EXERCISE **5-22** Lysine Iron Agar 407
EXERCISE **5-23** Litmus Milk Medium 411
♦ **Antimicrobial Susceptibility Testing** 417
EXERCISE **5-24** Bacitracin, Novobiocin,
and Optochin Susceptibility Tests 417
♦ **Other Differential Tests** 423
EXERCISE **5-25** Blood Agar 423
EXERCISE **5-26** CAMP Test 429
EXERCISE **5-27** Coagulase and Clumping Factor Tests 433
EXERCISE **5-28** Motility Test 437
♦ **Multiple Test Systems** 441
EXERCISE **5-29** api 20 E Identification System for
Enterobacteriaceae and Other Gram-negative Rods 441
EXERCISE **5-30** EnteroPluri-*Test* (Enterotube® II) 449
♦ **Bacterial Unknowns Project** 457
EXERCISE **5-31** Bacterial Unknowns Project 457

SECTION **6** **Quantitative Techniques** **467**
EXERCISE **6-1** Standard Plate Count (Viable Count) 469
EXERCISE **6-2** Urine Culture 479
EXERCISE **6-3** Direct Count (Petroff-Hausser) 483
EXERCISE **6-4** Closed-System Growth 487
EXERCISE **6-5** Plaque Assay of Virus Titre 497
EXERCISE **6-6** Thermal Death Time Versus Decimal Reduction Value 507

SECTION 7 **Medical Microbiology** 517

EXERCISE 7-1 Snyder Test 519
EXERCISE 7-2 Lysozyme Assay 523
EXERCISE 7-3 Antimicrobial Susceptibility Test: Disk Diffusion (Kirby-Bauer) Method 529
EXERCISE 7-4 Clinical Biofilms 537
EXERCISE 7-5 Morbidity and Mortality Weekly Report (MMWR) Assignment 541
EXERCISE 7-6 Epidemic Simulation 551
EXERCISE 7-7 Identification of *Enterobacteriaceae* 557
EXERCISE 7-8 Identification of Gram-positive Cocci 565
EXERCISE 7-9 Identification of Gram-positive Rods 573

SECTION 8 **Environmental Microbiology** 581

♦ **A Word About Trophic Groups** 582
EXERCISE 8-1 Winogradsky Column 583
♦ **Introduction to the Nitrogen Cycle** 589
EXERCISE 8-2 Nitrogen Fixation 591
EXERCISE 8-3 Nitrification: The Production of Nitrate 599
EXERCISE 8-4 Ammonification 605
EXERCISE 8-5 Denitrification: Nitrate Reduction 609
♦ **Sulfur Cycle—Introduction** 613
EXERCISE 8-6 Photosynthetic Sulfur Bacteria 615
EXERCISE 8-7 Chemolithotrophic Sulfur-Oxidizing Bacteria 621
EXERCISE 8-8 Sulfur-Reducing Bacteria 627
EXERCISE 8-9 Bioluminescence 633
EXERCISE 8-10 Soil Slide Culture 637
EXERCISE 8-11 Soil Microbial Count 641
♦ **Water Quality Testing** 647
EXERCISE 8-12 Membrane Filter Technique 647
EXERCISE 8-13 Multiple Tube Fermentation Method for Determining Water Quality 655

SECTION 9 **Food Microbiology** 665

EXERCISE 9-1 Methylene Blue Reductase Test 667
EXERCISE 9-2 Making Yogurt 671
EXERCISE 9-3 Yogurt CFU Density of an Active and Live Culture and Identification of a Yogurt Unknown 675

SECTION 10 **Microbial Genetics** 685

EXERCISE 10-1 Extraction of DNA from Bacterial Cells 687
EXERCISE 10-2 Restriction Digest 693
EXERCISE 10-3 Bacterial Transformation: The pGLO™ System 705
EXERCISE 10-4 Polymerase Chain Reaction 713
EXERCISE 10-5 Ultraviolet Radiation Damage and Repair 727
EXERCISE 10-6 Ames Test 733
EXERCISE 10-7 Phage Typing of *E. coli* Strains 739

SECTION 11 **Hematology and Serology** 743

EXERCISE 11-1 Differential Blood Cell Count 745
◆ **Introduction to Antigens and Antibodies** 751
EXERCISE 11-2 Precipitin Ring Test 753
EXERCISE 11-3 Double Gel Immunodiffusion 757
EXERCISE 11-4 Slide Agglutination 761
EXERCISE 11-5 Blood Typing 765
EXERCISE 11-6 ELISA for Detecting Antibodies in a Patient's Sample (The Antibody Capture Method) 771

SECTION 12 **Eukaryotic Microbes** 781

◆ **Eukaryotic Supergroups—A Refresher** 782
EXERCISE 12-1 Representative Fungi of Medical and Economic Importance 783
Division Zygomycota 785
Division Ascomycota 787
EXERCISE 12-2 Fungal Slide Culture 795
EXERCISE 12-3 Examination of Simple Eukaryotic Pathogens 801
Excavate Pathogens 801
Alveolate Pathogens 803
Unikont Pathogen 806
EXERCISE 12-4 Parasitic Helminths 813
Trematode Parasites Found in Clinical Specimens 813
Cestode Parasites Found in Clinical Specimens 815
Nematode Parasites Found in Clinical Specimens 818
Hookworms (*Ancylostoma duodenale* and *Nector americanus*) 820

APPENDIX A **Biochemical Pathways** 827
Oxidation of Glucose: Glycolysis, Entner-Doudoroff and Pentose-Phosphate Pathways 827
Oxidation of Pyruvate: The Krebs Cycle and Fermentation 830

APPENDIX B **Miscellaneous Transfer Methods** 835
Transfers Using a Sterile Cotton Swab 835
Stab Inoculation of Agar Tubes Using an Inoculating Needle 836
Spot Inoculation of an Agar Plate 837

APPENDIX C **Transfer from a Broth Culture Using a Glass Pipette** 839
Filling a Glass Pipette 840
Inoculation of Broth Tubes with a Pipette 841
Inoculation of Agar Plates with a Pipette 842

APPENDIX D **Transfer from a Broth Culture Using a Digital Pipette** 843
Filling a Digital Pipettor 843
Inoculation of Broth Tubes with a Digital Pipettor 844
Inoculation of Agar Plates with a Pipettor 845
Modifications for Transferring BSL-2 Microorganisms 846

APPENDIX E **The Spectrophotometer** 847
Theory 847
Instructions for Using the Spectronic D20+ 849

APPENDIX F **Alternative Procedures for Section 6** 851

Alternative Procedure for Exercise 6-1, Standard Plate Count (Viable Count) 851
Alternative Procedure for Exercise 6-5, Plaque Assay of Virus Titre. 853
Alternative Procedure for Exercise 6-6, Thermal Death Time Versus Decimal Reduction Value 855

APPENDIX G **Agarose Gel Electrophoresis** 857

Theory 857
Module A—Gel Preparation 858
Module B—Loading and Running Samples 859
Module C—Gel Staining and Destaining 861

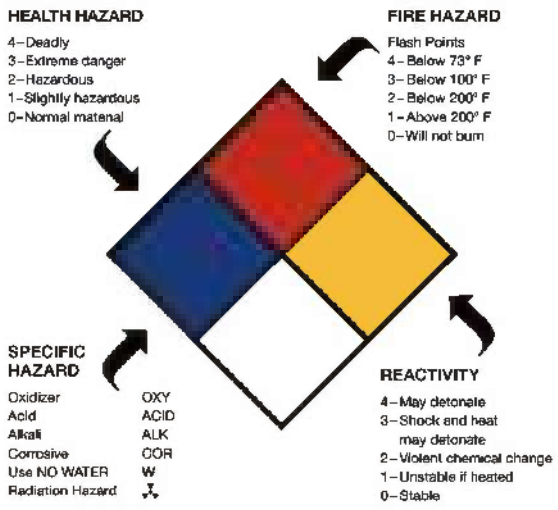
APPENDIX H **Medium, Reagent, and Stain Recipes** 863

Media 863
Reagents 876
Solutions 876
Stains 877
Gram Stain Procedure 878

Glossary 881

Index 889

Safety and Laboratory Guidelines



Microbiology lab can be an interesting and exciting experience, but at the outset you should be aware of some potential hazards. Improper handling of chemicals, equipment, and/or microbial cultures is dangerous and can result in injury or infection. Safety with lab equipment will be addressed when you first use that specific piece of equipment, as will specific examples of chemical safety. Our main concern here is to introduce you to safe handling and disposal of microbes.[1]

Because microorganisms present varying degrees of risk to laboratory personnel (students, technicians, and faculty), people outside the laboratory, and the environment, microbial cultures must be handled safely. The classification of microbes into four biosafety levels (BSLs) provides a set of minimum standards for laboratory practices, facilities, and equipment to be used when handling organisms at each level. These biosafety levels, defined in the U.S. government publication, *Biosafety in Microbiological and Biomedical Laboratories*, 5th edition (2009), are summarized below and in Table I-1. For complete information, readers are referred to the original document.

BSL-1: Organisms do not typically cause disease in healthy individuals and present a minimal threat to the environment and lab personnel. Standard microbiological practices are adequate. These microbes may be handled in the open, and no special containment equipment is required. Examples include *Bacillus subtilis*, *Escherichia coli* (most strains), *Rhodospirillum rubrum*, and *Lactobacillus acidophilus*.

BSL-2: Organisms are commonly encountered in the community and present a moderate environmental and/or health hazard. These organisms are associated with a variety of human diseases, most of which can be successfully treated if identified in a timely manner. The infection routes of primary concern are ingestion, inhalation, or penetration of the skin (percutaneous). Individuals performing work prone to splashes or aerosol generation (even though these organisms are not generally known to be transmitted by aerosols) should work in a biological safety cabinet (BSC). Otherwise, laboratory work may be done using standard microbiological practices. Examples include *Salmonella*, *Staphylococcus aureus*, *Clostridium dificile*, and *Borrelia burgdorferi*.

BSL-3: Organisms are of local or exotic origin and are associated with respiratory transmission and serious or lethal diseases where treatment and/or vaccines may or may not be available. Special ventilation systems are used to prevent aerosol transmission out of the laboratory, and access to the lab is restricted. Specially trained personnel handle microbes in a Class I or II BSC, not on the open bench (Fig. I.1). Examples include *Bacillus anthracis*, *Mycobacterium tuberculolosis*, and West Nile virus.

[1] Your instructor may augment or revise these guidelines to fit the conventions of your laboratory.

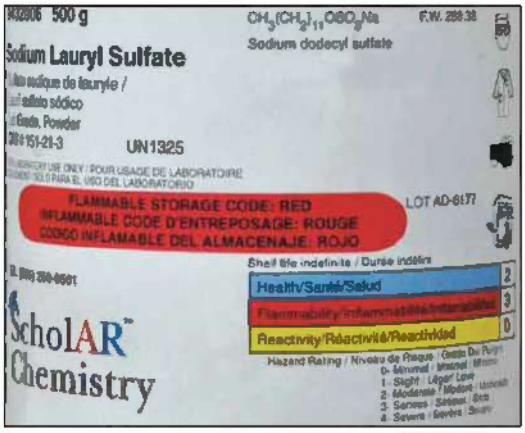
TABLE I-1 Summary of Recommended Biosafety Levels for Infectious Agents

BSL	Agents	Practices	Safety Equipment (Primary Barriers)	Facilities (Secondary Barriers)
1	■ Not known to consistently cause disease in healthy adults ■ e.g., *Lactobacillus casei, Bacillus subtilis, Rhizobium leguminosarum*	■ Standard microbiological practices	■ Primary barriers: none required ■ Personal Protective Equipment (PPE): laboratory coats and gloves; eye, face protection as needed	■ Laboratory bench practices and sink required
2	■ Agents associated with human disease; treatments and/or vaccines are usually available ■ Routes of transmission include percutaneous injury, ingestion, and mucous membrane exposure ■ e.g., *Staphylococcus aureus, Salmonella enterica*, measles virus	**BSL-1 practices plus:** ■ Limited lab access ■ Biohazard warning signs ■ "Sharps" precautions ■ Biosafety manual defining any needed waste decontamination	■ Primary barriers: Class I or II BCSs or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials ■ PPEs: laboratory coats, gloves, face protection as needed	**BSL-1 plus:** ■ Autoclave available
3	■ Indigenous or exotic agents with potential for aerosol transmission ■ Disease may have serious or lethal consequences; treatments and/or vaccines may be available ■ e.g., *Yersinia pestis, Mycobacterium tuberculosis*, rabies virus	**BSL-2 practices plus:** ■ Controlled lab access ■ Decontamination of all waste ■ Decontamination of all lab clothing before laundering ■ Baseline serum	■ Primary barriers: Class I or II BCSs or other physical containment devices used for all manipulations of agents ■ PPEs: protective lab clothing, gloves, respiratory protection as needed	**BSL-2 plus:** ■ Physical separation from access corridors ■ Access to self-closing double door ■ Exhausted air not recirculated ■ Negative airflow into laboratory
4	■ Dangerous/exotic agents that pose high risk of life-threatening disease, aerosol-transmitted lab infections, or related agents with unknown risk of transmission; treatment and vaccines are unavailable ■ e.g., Ebola virus, Lassa virus	**BSL-3 practices plus:** ■ Clothing change before entering ■ Shower on exit ■ All material decontaminated on exit from facility	■ Primary barriers: all procedures conducted in Class III BSCs or Class I or II BSCs *in combination with* full-body, air-supplied, positive pressure personnel suit	**BSL-3 plus:** ■ Separate building or isolated zone ■ Dedicated supply and exhaust vacuum, and decon system ■ Other requirements outlined in the text

Source: Adapted from Biosafety in *Microbiological and Biomedical Laboratories*, 5th edition (Washington, DC: U.S. Government Printing Office, 2007).

I.1 Biological Safety Cabinet in a BSL-2 Laboratory ♦ In this Class II BSC, air is drawn in from the room and is passed through a HEPA filter prior to release into the environment. This airflow pattern is designed to keep aerosolized microbes from escaping from the cabinet. The microbiologist is pipetting a culture. When the BSC is not in use at the end of the day, an ultraviolet light is turned on to sterilize the air and the work surface.

(San Diego County Public Health Laboratory)



I.2 Chemical Hazard Label ♦ The blue, red, and yellow boxes on this label indicate the health, flammability, and reactivity levels, respectively, of the compound sodium dodecyl sulfate (SDS). The colors are standardized (regardless of the chemical) and each is assigned a number between "0" (minimal) and "4" (severe) as shown in the legend at the lower right. SDS presents a moderate health hazard, is very flammable, but minimally reactive. Also notice the icons in the upper right, which indicate (from top to bottom) that this compound should be handled with eye protection, a lab coat, gloves, and adequate ventilation. Be aware of these warnings and comply with the associated cautions for all chemicals you handle in lab.

BSL-4: Organisms have a great potential for lethal infection. Inhalation of infectious aerosols, exposure to infectious droplets, and autoinoculation are of primary concern. The lab is isolated from other facilities, and access is strictly controlled. Ventilation and waste management are under rigid control to prevent release of the microbial agents to the environment. Specially trained personnel perform transfers in Class III BSCs. Class II BSCs may be used as long as personnel wear positive pressure, one-piece body suits with a life-support system. Examples include agents causing hemorrhagic diseases, such as Ebola virus, Marburg virus, and Lassa fever.

The microorganisms used in introductory microbiology courses depend on the institution, objectives of the course, and student preparation. Most introductory courses use organisms that may be handled at BSL-1 and BSL-2 levels so we have followed that practice in designing this set of exercises. Following are general safety rules to reduce the chance of injury or infection to you and to others, both inside and outside the laboratory. Although both BSL-1 and BSL-2 guidelines are listed, we believe it is best to err on the side of caution and that students should learn and practice the safest level of standards (relative to the organisms they are likely to encounter) at all times. Please follow these and any other safety guidelines required by your college.

Chemical safety is also important in a microbiology laboratory. Be aware of the hazards presented by the chemicals you are handling. Containers should be labeled with a standard set of precautions as seen in Figure I.2. Numbers are assigned to the degree of health, fire, and reactivity hazard posed by the chemical. On stickers placed by the laboratory, there also is a space to enter specific hazards, such as acid, corrosive, and radioactivity.

Student Conduct

- To reduce the risk of infection, do not smoke, eat, drink, or bring food or drinks into the laboratory room—even if lab work is not being done at the time.
- Do not apply cosmetics or handle contact lenses in the laboratory.
- Wash your hands *thoroughly* with soap and water after handling living microbes and before leaving the laboratory each day. Also, wash your hands after removing gloves.
- Do not remove any organisms or chemicals from the laboratory.
- Lab time is precious, so come to lab prepared for that day's work. Figuring out what to do as you go is likely to produce confusion and accidents.
- Work carefully and methodically. Do not hurry through any laboratory procedure.

Basic Laboratory Safety

- Wear protective clothing (i.e., a lab coat) in the laboratory when handling microbes. Remove the coat prior to leaving the lab and autoclave it regularly (Fig. I.3).
- Wear only closed-toe shoes in the laboratory.
- Wear eye protection whenever you are heating chemicals, even if you wear glasses or contacts (Fig. I.3).
- Turn off your Bunsen burner when it is not in use. In addition to being a fire and safety hazard, it is an unnecessary source of heat in the room.
- Tie back long hair, because it is a potential source of contamination as well as a likely target for fire.
- If you are feeling ill, go home. A microbiology laboratory is not a safe place if you are ill.
- If you are pregnant, immune compromised, or are taking immunosuppressant drugs, please see the instructor. It may be in your best long-term interests to postpone taking this class. Discuss your options with your instructor.
- If it is your lab's practice to wear disposable gloves while handling microorganisms, be sure to remove them each time you leave the laboratory. The proper method for removal is with the thumb under the cuff of the other hand's glove and turning it inside out without snapping it. Gloves should then be disposed of in the container for contaminated materials. Then, wash your hands.
- Wear disposable gloves while staining microbes and handling blood products—plasma, serum, antiserum, or whole blood (Fig. I.3). Handling blood can be hazardous, even if you are wearing gloves. Consult your instructor before attempting to work with any blood products.
- Use an antiseptic (e.g., Betadine®) on your skin if it is exposed to a spill containing microorganisms. Your instructor will tell you which antiseptic you will be using.
- Never pipette by mouth. Always use mechanical pipettors (Fig. C-1, Appendix C).
- Dispose of broken glass or any other item that could puncture an autoclave bag (contaminated or not) in an appropriate sharps or broken glass container (Fig. I.4).
- Use a fume hood to perform any work involving highly volatile chemicals or stains that need to be heated.
- Find the first-aid kit, and make a mental note of its location.

I.3 Safety First ♦ This student is prepared to work safely with microorganisms. The lab area is uncluttered, tubes are upright in a test tube rack, and the flame is accessible but not in the way. The student is wearing a protective lab coat, gloves, and goggles, all of which are to be removed prior to leaving the laboratory. Not all procedures require gloves and eye protection. Your instructor will advise you as to the standards in your laboratory.

I.4 Sharps Container ♦ Needles, glass, and other contaminated items that can penetrate the skin or an autoclave bag should be disposed of in a sharps container. Do not fill above the dashed black line. Notice the autoclave tape in the lower left. The white stripes will turn black after proper autoclaving. Above the autoclave tape is the address of the institution that produced the biohazardous waste.

- Find the fire blanket, shower, and fire extinguisher, note their locations, and develop a plan for how to access them in an emergency.
- Find the eyewash basin, learn how to operate it, and remember its location.

Reducing Contamination of Self, Others, Cultures, and the Environment

- Wipe the desktop with a disinfectant (e.g., Coverage® or 10% chlorine bleach) before *and* after each lab period. Never assume that the class before you disinfected the work area. An appropriate disinfectant will be supplied. Allow the disinfectant to evaporate; do not wipe it dry.
- Never lay culture tubes on the table; they always should remain upright in a tube holder (Fig. I.3). Even solid media tubes contain moisture or condensation that may leak out and contaminate everything it contacts.
- Cover any culture spills with paper towels. Soak the towels immediately with disinfectant and allow them to stand for 20 minutes. Report the spill to your instructor. When you are finished, place the towels in the container designated for autoclaving.
  - If the culture spills on you, remain where you are, don't touch anything, and have your lab partner notify the instructor. Your instructor will advise your group on how to handle the spill.
  - If you get a microbial culture in your eyes IMMEDIATELY have a lab partner lead you to the eyewash basin and rinse your eyes for at least 15 minutes. Time is of the essence for eye contamination. Your lab partners must notify the instructor of the spill and your situation, and follow his/her instructions for handling your care and the spill.
- Place all nonessential books and papers under the desk. A cluttered lab table is an invitation for an accident that may contaminate your expensive school supplies.
  - Cell phones, tablets, computers, and other electronic devices must never be on the lab table when working with microbes. Contamination of these items can be a health hazard as well as expensive.
- When pipetting microbial cultures, place a disinfectant-soaked towel on the work area. This reduces contamination and possible aerosols if a drop escapes from the pipette and hits the tabletop.

Guidelines Governing Handling of BSL-2 Organisms

Following is a list of precautions for handling BSL-2 organisms. A number of these were already listed as optional, but are repeated here along with the new ones and now are considered "highly recommended." It is also highly recommended that before students are allowed to handle BSL-2 organisms, they should demonstrate skill in handling BSL-1 organisms safely.

Your college may have other guidelines for handling BSL-2 organisms and other standards of practice may be adopted during the lifetime of this book, so pay attention to any announcements your lab instructor may give. And, as always, it is the instructor's responsibility to choose organisms and lab exercises appropriate to the skill level of his/her students.

- The lab instructor should announce to the class when BSL-2 precautions are in effect. Your college may also institute a mechanism for identifying BSL-2 cultures, such as using red caps on culture tubes. Be aware of any conventions used by your lab.
- Access to the lab must be limited once work with the BSL-2 organisms has begun. Doors and windows must be closed.
- All unnecessary materials (books, backpacks, etc.—in other words, anything you plan on taking home) must be kept off the work surface and safe from aerial contamination and spills. Your lab will have a designated place for their storage.
- Disinfect your lab table top before and after the activity. Allow the disinfectant to air-dry.
- A lab coat, gloves, and eye protection must be worn throughout the handling of BSL-2 organisms. Do not touch any items not directly involved in the lab activity. Properly remove and dispose of the gloves when finished. (See page 4 for proper glove removal.)
- Writing utensils must be "lab dedicated," that is, they do not leave the lab until they have been disinfected. It is best to wipe them down with disinfectant after use but keep them in your lab drawer for the entire semester. There is no sense in risking contamination of multiple pens and pencils by using different ones each day.
- Electronic devices (e.g., laptops, tablets, cell phones) must be kept off the work surface and stored in an area protected from spills and aerial contamination.

- Use page covers to protect your lab exercise pages while performing the activity. Once your work is completed, disinfect the page covers, wash your hands, and remove the pages. When dry, the page covers can be stored in your locker and reused. The lab book pages can be taken home.
- It is best to use a photocopy of your data sheet to record results. Once you have decontaminated the area, it can be photographed and the results can be transcribed to the original data sheet. Dispose of the photocopy in an appropriate biohazard container. It must not leave the lab.
- Minimizing aerosol production during the transfer of microorganisms is important and the instructions in Section 1 address methods for doing so. However, here is a summary of recommended practices specific to BSL-2 organisms.
  - Wire loops and needles should be incinerated in an enclosed electric incinerator (Fig. 1.14). Sterilization with an open flame should be avoided because of its potential for aerosol production.
  - An alternative to the electric incinerator is to perform transfers with sterile disposable loops or sterile wooden sticks. Both will work for transferring from solid or liquid media. After each use, they should be put in a disposal container (such as a can) contaminated end down in about 2 cm of disinfectant. When finished with the exercise, the can should be put in a bin for autoclaving or other disposal receptacle your lab uses. Alternatively, sticks and disposable loops can be put in a sharps container if that is your lab's practice.
  - Pipetting must be done in such a way that the last drop is *not* "blown out" unless a Petri dish lid is covering a plate. Appendices C and D give specific instructions for pipetting BSL-2 organisms.
- Clean up after the activity.
  - For incubation, place tubed cultures in a rack. For plate cultures, it is best to tape the lids on each plate individually by taping around the edge of the plate rather than taping around the base and lid (Fig. I.5). This allows viewing of the agar surface without having to remove the tape.
  - Wipe down the work surface, writing utensils, and page covers (if any) with disinfectant and let them air-dry. Only after the disinfectant has dried is it OK to place items to be removed from lab on your table.
  - Remove your gloves, eye protection, and lab coat and store them according to your lab's conventions.
  - Wash your hands thoroughly.
  - Photograph your data sheet and dispose of it properly.

Disposing of Contaminated Materials

In most instances, the preferred method of decontaminating microbiological waste and reusable equipment is the autoclave (Fig. I.6).

- Remove all labels from tube cultures and other contaminated *reusable* items and place them in the designated autoclave container. This will likely be an open autoclave pan to enable cleaning the tubes, and other items following sterilization.

I.5 Taping Plates ♦ When taping plate lids, it is best to run the tape around the lid's edge, and then press it firmly onto the base, as shown. Taping across the lid and base obscures the agar and makes reading a plate more difficult. This is especially problematic when BSL-2 organisms are being grown and the lid needs to remain on the plate.

I.6 An Autoclave ♦ Media, cultures, and equipment to be sterilized are placed in the basket of the autoclave. Steam heat at a temperature of 121°C (produced at atmospheric pressure plus 15 psi) for 15 minutes is effective at killing even bacterial spores. Some items that cannot withstand the heat, or have irregular surfaces that prevent uniform contact with the steam, are sterilized by other means.

- Dispose of plate cultures (if plastic Petri dishes are used) and other contaminated non-sharp *disposable* items in the autoclave container so designated (Fig. I.7). Petri dishes should be taped closed. (***Note:*** To avoid recontamination of sterilized culture media and other items, autoclave containers are designed to be permanently closed, autoclaved, and discarded. Therefore, do not place reusable and nonreusable items in the same container.
- Dispose of all blood product samples and disposable gloves in the container designated for autoclaving.
- Place used microscope slides of bacteria in a sharps container designated for autoclaving, or soak them in disinfectant solution for at least 30 minutes before cleaning or discarding them. Follow your laboratory guidelines for disposing of glass.
- Place contaminated broken glass and other sharp objects (anything likely to puncture an autoclave bag) in a sharps container designated for autoclaving (Fig. I.4). Uncontaminated broken glass does not need to be autoclaved, but should be disposed of in a specialized broken glass container.

I.7 An Autoclave Bag ◆ Nonreusable items (such as plastic Petri dishes) are placed in an autoclave bag for decontamination. Petri dishes should be taped closed. Do not overfill or place sharp objects in the bag. Notice the autoclave tape at the middle right. The white stripes will turn black after proper autoclaving. At the lower right is the address of the institution that produced the biohazardous waste.

Student Compliance with Laboratory Safety Regulations

Your institution may augment the safety regulations outlined above or compile a list tailored to your laboratory. In either case, these constitute a Laboratory Safety Statement. It is highly recommended that students verify that they have read and understand these safety regulations, agree to comply with them, and are aware of noncompliance consequences by signing a statement to that effect. The American Society for Microbiology has produced a publication (Emmert et al., 2012) that includes a sample Laboratory Safety Statement and a student signature page. The signature portion in a slightly modified form is reproduced on page 13. Your instructor may have you sign it or sign his/her own version of it.

References

Barkley, W. Emmett and John H. Richardson. Chap. 29 in *Methods for General and Molecular Bacteriology*, Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Chaps. 1 and 4 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, UK: Butterworth-Heineman, 1995.

Darlow, H. M. Chap. VI in *Methods in Microbiology*, Volume 1, J. R. Norris and D. W. Ribbins, eds. London, UK: Academic Press, Ltd., 1969.

Emmert, Elizabeth A. B., Jeffrey Byrd, Ruth A. Gyure, Diane Hartman, and Amy White et al. "Biosafety Guidelines for handling Microorganisms in the Teaching Laboratory: Development and Rationale." *Journal of Microbiology & Biology Education* (May 2013): 78–83. DOI: http://dx.doi.org/10.1128/jmbe.v14i1.531.

Emmert, Elizabeth A. B., Jeffrey Byrd, Ruth A. Gyure, Diane Hartman, and Amy White et al. *Appendix to the Guidelines for Biosafety in Teaching Laboratories*. Washington, DC: American Society for Microbiology, 2012.

Fleming, Diane O. and Debra L. Hunt, eds. *Laboratory Safety—Principles and Practices*, 3rd ed. Washington, DC: American Society for Microbiology, 2000.

Koneman, Elmer W., Stephen D. Allen, William M. Janda, Paul C. Schreckenberger, and Washington C. Winn, Jr. *Color Atlas and Textbook of Diagnostic Microbiology*, 5th ed. Philadelphia and New York: Lippincott-Raven Publishers, 1997.

Power, David A. and Peggy J. McCuen. Pages 2–3 in *Manual of BBL™ Products and Laboratory Procedures*, 6th ed. Cockeysville, MD: Becton Dickinson Microbiology Systems, 1988.

Wilson, Deborah E. and L. Casey Chosewood. U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. Washington, DC: U.S. Government Printing Office, 2007.

A Word About Experimental Design

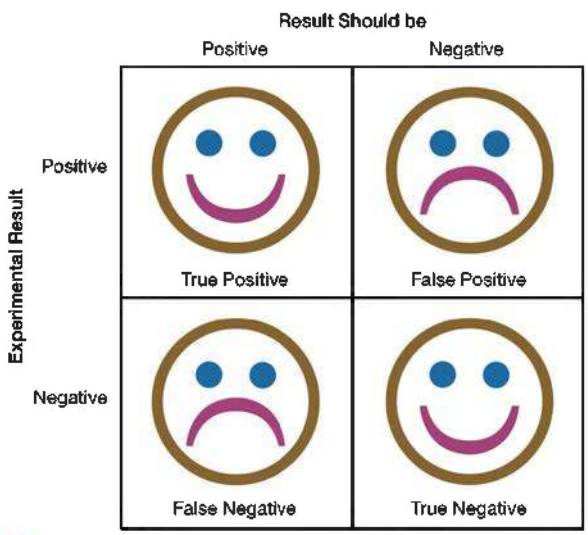
Like most sciences, microbiology has descriptive and experimental components. Here we are concerned with the latter. Science is a philosophical approach to finding answers to questions. Despite what you may have been taught in grade school about *THE* "Scientific Method," science can approach problems in many ways, rather than in any *single* way. The nature of the problem, personality of the scientist, intellectual environment at the time, and good, old-fashioned luck all play a role in determining which approach is taken. Nevertheless, in experimental science, one component that is always present is a **control** (or controls).

A controlled experiment is one in which all **variables** except one—the **experimental variable**—are maintained without change. Frequently, we are looking for the cause of some event, activity, or function. By limiting variables to only one we can draw a provisional conclusion about whether or not that variable causes the event. If changing the variable causes a change in the event, then we can provisionally conclude that the variable and the event are causally related; that is, we have demonstrated a **cause and effect relationship** between the event and the experimental variable. Alternatively, if there is no observed change, we can eliminate the experimental variable from involvement with the event.

For example, if we want to determine the effect of increasing temperature on microbial growth rate, we could grow the same microorganism in two test tubes containing the same nutrient source at two different temperatures. One would be grown at "normal" temperature (whatever it is for that organism) and the other would be grown at a higher temperature. Everything but temperature would be the same, so that if we see a difference in growth rate we can attribute it to the experimental variable, temperature. But how would we know there is a difference in growth rate? Simple—by comparing the growth rate at the higher temperature to the growth rate at the "normal" temperature. The "normal" temperature tube is the control; it provides a baseline growth rate against which the experimental growth rate is compared. Growth rate in the experimental tube will be faster, slower, or the same as the control. Without the control we would only be able to measure growth rate in the experimental tube, but we couldn't answer the question about the effect of higher temperature on growth rate. Had we grown different microorganisms with different nutrient sources at different temperatures and we saw a difference, we wouldn't know if the difference was due to the organism, the nutrients available, and/or the temperature. This is why we test one variable at a time.

Controls are an essential and integral part of all experiments, but there are many types. Two commonly used controls are **positive** and **negative controls.** A positive control is one that is set up to produce a positive result. Alternatively, a negative control is set up to demonstrate a negative result or no change. As you work your way through the exercises in this book, pay attention to the various ways positive, negative, and other controls are used to improve the **validity** (effectiveness) of the experiment and the **reliability** (accuracy) of the results. In fact, there are many questions on the data sheets that ask some form of the question: "What is the role of the control in this experiment?" We are pretty sure that your professor won't just be satisfied with the answer, "It's a control."

Microbiological experimentation often involves tests that determine the ability of an organism to use or produce some chemical, or to determine the presence or absence of a specific organism in a sample. Ideally, a positive result in the test indicates that the microbe has the ability or is present in the sample, and a negative result indicates a lack of that ability or absence in the sample (Fig. I.8). The tests we run, however, have limitations and occasionally may give **false positive** or **false negative** results. An inability to detect small amounts of the chemical or organism in question would yield a false negative result and would be the result of inadequate **sensitivity** of the test (Fig. I.8). An inability to discriminate between the chemical or



I.8 Limitations of Experimental Tests ♦ Ideally, tests should give a positive result for specimens that are positive, and a negative result for specimens that are negative. False positive and false negative results do occur, however, and these are attributed to inadequate specificity and inadequate sensitivity, respectively, of the test system.

organism in question and similar chemicals or organisms would yield a false positive result and would be the result of inadequate specificity of the test (Fig. I.8). Sensitivity and specificity can be quantified using the following equations:

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}}$$

$$\text{Specificity} = \frac{\text{True Negatives}}{\text{True Positives} + \text{False Negatives}}$$

The closer sensitivity and specificity are to a value of one, the more useful the test is. As you perform the tests in this book, be mindful of each test's limitations, and be open to the possibility of false positive and false negative results.

References

Forbes, Betty A., Daniel F. Sahm, and Alice. S. Weissfeld. Chap. 5 in *Bailey & Scott's Diagnostic Microbiology*, 10th ed. St. Louis, MO: Mosby-Year Book, 1998.

Lilienfeld, David E. and Paul D. Stolley. Page 118 in *Foundations of Epidemiology*, 3rd ed. New York: Oxford University Press, 1994.

Mausner, Judith S. and Shira Kramer. Pages 217–220 in *Epidemiology: An Introductory Text*, 2nd ed. Philadelphia: W.B. Saunders Company, 1985.

Data Presentation: Tables and Graphs

In microbiology, we perform experiments and collect data, but it is often difficult to know what the data mean without some method of organization. Tables and graphs allow us to summarize data in a way that makes interpretation easier.

Tables

A table is often used as a preliminary means of organizing data. As an example, Table I-2 shows the winning times for each male and female age division in a half-marathon race. Again, the aim of a table is to provide information to the reader. Notice the meaningful title, the column labels, and the appropriate measurement units. Without these, the reader cannot completely understand the table and your work will go unappreciated! Data tables are provided for you on the data sheets for each exercise in this book, but you may be required to fill-in certain components (units, labels, etc.) in addition to the data.

Graphs

Table I-2 does give the information, but what it is telling us may not be entirely clear. It appears that the times increase as runners get older, but we have difficulty determining if this is truly a pattern. That is why data also are presented in graphic form at times; a graph usually shows the relationship between variables better than a table of numbers.

TABLE I-2 Winning Half-Marathon Times by Sex and Age Division

Male Runners		Female Runners	
Winner's Age (years)	Winning Time (minutes)	Winner's Age (years)	Winning Time (minutes)
15	73	15	88
25	67	24	82
31	67	30	82
35	71	39	84
40	71	42	85
52	78	50	109
62	95	62	108
70	123	70	126

X–Y Scatter Plot The type of graph you will be using in this manual is an "X–Y Scatter Plot," in which two variables are graphed against each other. Figure I.9 shows the same data as Table I-2, but in an X–Y Scatter Plot form.

Notice the following important features of the graph in Figure I.9:

- *Title:* The graph has a meaningful title—which should tell the reader what the graph is about. A title of "Age vs. Winning Time" is vague and inadequate.
- *Dependent and independent variables:* The graph is read from left to right. In our example, we might say for the male runners, "As runners get older, winning times get longer." *Winning time* depends on *age*, so winning time is the *dependent* variable and age is the *independent* variable. By convention, the independent variable is plotted on the *x*-axis and the dependent variable is plotted on the *y*-axis. (Age does *not* depend on the winning time.) By way of comparison, notice the consequence of plotting age on the *y*-axis and winning time on the *x*-axis: "As runners get slower, they get older"—which doesn't reflect the actual relationship between the variables and worse yet, is nonsense.
- *Axis labels:* Each axis is labeled, including the appropriate units of measure. "Age" without units is meaningless. Does the scale represent months? years? centuries?
- *Axis scale:* The scale on each axis is uniform. The distance between marks on the axis is always the same and represents the same amount of that variable. (But increments on the *x*-axis don't have to equal those on the *y*-axis, as shown.) The size of each increment

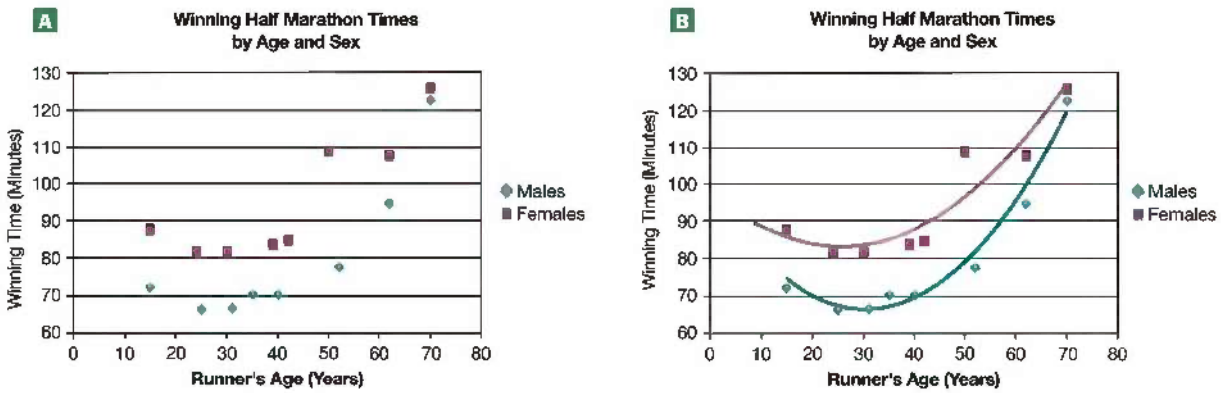
is up to the person making the graph and is dictated by the magnitude and range of the data. Most of the time, we choose a length for the axis that fills the available graphing space.

- *Axis range:* The scale for an axis does not have to begin with "0." Use a scale that best presents the data. In this case, the smallest *y*-value was 67 minutes, so the scale begins at 60 minutes.
- *Multiple data sets and the legend:* The two data series (male and female times) are plotted on the same set of axes, but with different symbols that are defined in the legend at the right. The symbols shown differ in color *and* shape, but one of these is adequate.
- *Best-fit line:* If a line is to be drawn at all, it should be an average line for the data points, not one that "connects the dots" (Fig. I.9B). Notice that the points are not necessarily *on* the line. The purpose of a best-fit line is to illustrate the general trend of the data, not the specifics of the individual data points. (Be assured that most graphs in your textbooks where a smooth line is shown were experimentally determined and the lines are derived from points scattered around the line.) There is a mathematical formula that allows one to compute the slope and *y*-intercept of the trend line if the relationship is linear or a best-fit line if the relationship is nonlinear (as in the half-marathon times example), but this is beyond our needs. For our purposes, a hand-drawn trend line that looks good is good enough. (If you use a computer graphing program, then it will produce the trend line without you doing any of the math—the best of all situations!)

Bar Graphs Bar graphs are used to illustrate one variable. Often, the variable is considered to be categorical. That is, the data are in distinct groups (rather than continuous, where the breaks are arbitrary). Using a bar graph to show the relationship between winning times and ages is inappropriate. Examine Figure I.10A. Notice that the space each bar fills is meaningless; that is, the only important part of the bar is the top—which is the value used in the *X*–*Y* scatter plot. Also notice that the *x*-axis scale is not uniform—the gaps between bars range from 4 years (31 to 35) to 12 years (40 to 52). An appropriate use of a bar graph would be the distribution of student performance on an exam, even though exam scores are not categorical (Fig. I.10B). Notice that the space each bar fills has meaning. Each student in a particular group adds height to the bar.

Data Presentation: Be Creative, But Complete

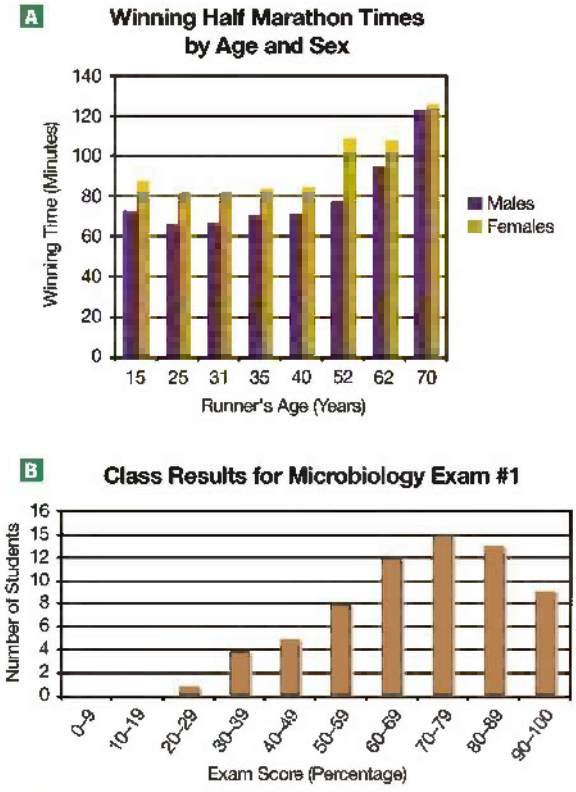
There is no single correct way to produce a graph for a particular data set. Actually, most people working independently would graph the same data set in different ways (e.g., different scales, colors, wording of the title, and axis labels), but the essential components listed would have to be there. You will be asked to graph some of the data you collect. Be sure your graphs tell a complete and clear story of what you have done.



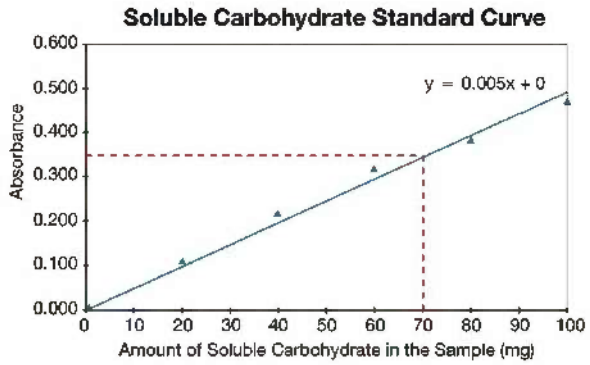
I.9 Sample X–Y Scatter Plot ♦ A graph often shows the relationship between variables better than a table of numbers. Examine this sample and identify the essential components of a quality graph (see text). **(A)** Presentation of data without a best-fit line is acceptable if there are not enough data points to justify illustrating a trend. **(B)** Shown here are the same data but with a trend line. Notice that the points do not fall directly on the line but, rather, that the line gives the general trend of the data. "Connecting the dots" is not appropriate.

Standard Curve Besides making interpretation easier, graphs sometimes are used to establish an experimental value. Each point on the trend line represents a correlation between the x and y values (Fig. I.11). If we know one value, we can read the other off the graph. We use this process with something called a "standard curve" or "calibration curve." To produce a standard curve, samples with a known amount of the independent variable (e.g., soluble carbohydrate concentration) are subjected to the experiment. The resulting data (the y values—e.g., absorbances) are plotted and a trend line is drawn.

Now a sample with an *unknown* amount of the independent variable can be subjected to the same experimental procedure to determine its y value. Once the y value is known, the corresponding x value is read directly off the graph to determine the unknown amount. (If the relationship is linear, the trend line is described by the equation $y = mx + b$, where m is the line's slope and b is the point at which the line intersects the y-axis. Once the y value is determined experimentally, the x value can be calculated by substitution.)



I.10 Bar Graphs ◆ A bar graph is appropriate to present data involving a single variable, especially if that variable is easily divided into distinct categories or groups. (**A**) Plotting the winning half-marathon times from Table I.2 using a bar graph is inappropriate because the only meaningful point is at the top. (**B**) A bar graph is useful in presenting data of a single variable, such as the number of students earning a specific score on their microbiology exam.



I.11 A Standard Curve ◆ Standard curves are used to determine the value of some unknown in a sample. In this example, the absorbances of six solutions with known soluble carbohydrate concentrations were determined experimentally, plotted (blue triangles), and used to make a trend line (blue line). Absorbance of a sample with an unknown soluble carbohydrate concentration then was determined to be 0.35 by the same experimental method. The point where the y value (ABS = 0.35) intersects the trend line gives the x value that corresponds to it (red dashed lines): 70 µg of soluble carbohydrate. The equation for the trend line ($y = 0.005x + 0$) is also given and can be used to determine the x value by substituting 0.35 for y.

Student Safety Contract

Student Agreement on Laboratory Safety

I have read the Laboratory Safety Statement as written in *Microbiology Laboratory Theory & Application,* 4th ed. and/or the equivalent document supplied by the Department of Biological Sciences, _____ (Institution Name),

and I understand its content. I agree to abide by all laboratory rules set forth by the instructor. I understand that my safety is entirely my own responsibility and that I may be putting myself and others in danger if I do not abide by all the rules set forth by the instructor.

COURSE: _____

STUDENT NAME (PRINT): _____

STUDENT SIGNATURE: _____

DATE: _____

SECTION 1

Fundamental Skills for the Microbiology Laboratory

A necessary skill for safely working in a laboratory, handling foods, and just living in a world full of microbes, is effective hand washing. In Exercise 1-1, you will have the opportunity to evaluate and compare hand-cleansing products.

A second skill microbiologists should at the very least be familiar with, and at most be able to do as a matter of routine, is prepare the **media** used to grow bacterial and fungal **cultures**. Media must be sterile prior to use and must also supply the organisms to be grown in or on them with the chemical nutrients necessary for the organism's growth. Preparation of these media involves weighing ingredients, measuring liquid volumes, calculating proportions, handling basic laboratory glassware, and operating a pH meter and an autoclave. In Exercise 1-2 you will learn and practice these fundamental skills by preparing a couple of simple growth media. When you have completed the exercise, you will have the skills necessary to prepare almost any medium if given the recipe.

A third fundamental skill necessary for any microbiologist is the ability to transfer microbes from one place to another without contaminating the original culture, the new medium, or the environment (including the microbiologist and others in the laboratory). This **aseptic** (without contamination) transfer technique is required for virtually all procedures in which living microbes are handled, including isolations, staining, and differential testing. Exercises 1-3 through 1-5 present descriptions of common transfer and inoculation methods. Less frequently used methods are covered in Appendices B through D.

EXERCISE
1-1

A Comparison of Hand-Cleansing Agents[1]

Theory

The concept of good hand hygiene has gone from a controversial beginning (in the early 1800s) to an accepted practice that is still problematic. Current studies designed to test the efficacy of various agents often have subjects wash for unrealistic lengths of time (that is, longer than workers routinely wash on the job), test artificially contaminated hands (or not), and use different standards of evaluation, making comparison difficult. We are still left with the question, "What works best?"

While it has been identified as an important, easily performed behavior that minimizes transfer of pathogens to others, uniform compliance with hand-washing standards has been difficult to achieve. Heavy workloads, skin reactions to the agent (e.g., plain or antimicrobial soap, iodine compounds, alcohol), skin dryness due to frequent washing, and many other factors contribute to noncompliance (see Boyce and Pittet, 2002, and The Joint Commission, 2009).

Alcohol-based hand rubs have, in many instances, replaced conventional hand-washing agents because they are more effective than soap and water (disputed), require less time, produce fewer skin reactions, and have been shown to result in a higher level of compliance by health-care workers. Examples of agents that can be tested in this exercise are shown in Figure 1.1.

Application

Effective hand washing to minimize direct person-to-person transmission, and indirect contact of pathogens by health-care professionals and food handlers, is essential. It also is critical to laboratorians handling pathogens to minimize transmission to others, inoculation of oneself, and contamination of cultures.

In This Exercise

You will evaluate the relative effectiveness of a variety of hand-washing agents.

Materials

Per Student

- ☐ One nutrient agar or tryptic soy agar plate
- ☐ Access to a sink and paper towels
- ☐ Permanent marking pen

[1] Thanks to reviewers Johana Melédez-Santiago and Janice Yoder Smith for their suggestions on developing this laboratory exercise.

1.1 Hand-Washing Agents ♦ From left to right are: Betadine® scrub (10% povidone iodine), alcohol-based hand sanitizer (65% ethyl alcohol), 0.13% benzalkonium chloride-based hand sanitizer, antibacterial soap (0.15% triclosan), and Hibiclens® (4% chlorhexidine gluconate). As written, this exercise uses these agents, but others can be substituted, including tap water.

1 Per Table

- ☐ Bottles of as many as five of the following or suitable replacements:
  - Antibacterial liquid soap (0.15% triclosan)
  - Hand sanitizer (65% ethyl alcohol = 65% ethanol)
  - Hy5 brand of alcohol-free hand sanitizer (0.13% benzalkonium chloride)
  - Betadine® scrub (10% povidone iodine)
  - Hibiclens® (4% w/v chlorhexidine gluconate)
  - Tap water

Note: Students should not ingest or get any of these products in their eyes. Follow package inserts for treatments. Students with allergies or sensitivities to the active ingredients in any of these products should be assigned an alternate product.

Procedure

Lab One

1. Label the base of a nutrient agar or tryptic soy agar plate with your name. Divide the plate in half and label one half "Before" and the other half "After." Then, number 1 through 5 on both sides of the dividing line so the numbers line up. That is, number 1 on the "Before" side should be opposite number 1 on the "After" side, and so on. Use the entire diameter of the plate for the five numbers and spread them out as much as you can. You will be pressing your fingers at each number in steps 3 and 6.
2. Rub your left hand fingertips (all five of them) on the area to be sampled. (Good choices are the floor, shoe soles, tabletop near a sink, backpack, and your forehead or feet. Be creative!
3. Then, one at a time, gently press your thumb tip next to the "Before" number 1, your index finger next to the "Before" number 2, and so on. Your middle, ring, and pinky fingers are numbered 3, 4, and 5, respectively.
4. Wash your hands with soap and water.
5. Record the hand-cleansing agents you will be using on the data sheet, page 19.
6. Now sample the same site used in step 2 with the fingertips of your right hand, but this time one fingertip at a time. Then, you will rub that fingertip in a few milliliters of the hand-cleansing agent in your left palm (see steps a and b for further directions). If the agent requires rinsing and drying, perform this at a sink. Agents that only require air-drying can be done at your table.
   a. Place a quarter-size drop of the first hand-cleansing agent in the palm of your left hand. Rub your right thumb on the area to be sampled. Then, rub your thumb into the agent for 10 seconds.[2] (If you don't have a watch, sing the "Happy Birthday Song" once to yourself.) Once your thumb is dry, gently press it next to the number 1 on the "After" side. Wash both hands with soap and water, and dry them.
   b. Repeat step a with each finger of the right hand and the remaining four hand-cleansing agents. Be sure to press each finger onto the agar next to its correct number and to wash your hands in between exposures.
7. Tape the lid on the plate and incubate at 25°C until the next lab period.

Lab Two

1. Examine your plates for growth. (*Note*: don't remove the plate's lid unless given permission to do so.) Each "blob" is probably the product of a single microbial species that has reproduced from one or a few cells to such an extent that it is now visible to the naked eye. This is called a "colony." Different microbial species often produce distinctly different colonies.
2. Fill in the table provided on the data sheet, page 19, as you evaluate the relative amount of growth and microbial diversity (based on colony differences) on the "Before" and "After" sides of the plate for each agent. Recognize that you are comparing "apples and oranges" with respect to their intended uses. That is, the products tested range from surgical scrubs to over-the-counter soaps intended for household use. A surgical scrub may have better antimicrobial activity than hand soap, but that doesn't make it "better" for household use!

[2] The recommended time for hand washing is 15–20 seconds, but because you are only washing the fingertips and concentrating on those, that time has been reduced in order to see representative results.

References

Boyce, John M. and Didier Pittet. Centers for Disease Control and Prevention. *Guideline for Hand Hygiene in Health-Care Settings: Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force.* MMWR 2002, 51 (No. RR-16): 1-45.

The Joint Commission, 2009. *Measuring Hand Hygiene Adherence: Overcoming the Challenges.* http://www.jointcommission.org/assets/1/18/hh_monograph.pdf.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 1-1

A Comparison of Hand-Cleansing Agents

OBSERVATIONS AND INTERPRETATIONS

1 Record your results in the table below. For **relative amount of growth**, use this qualitative scale for evaluation:

+++ means "a lot of contamination"
++ means "moderate contamination"
+ means "little contamination"
0 means "no contamination"

There is no absolute cutoff between any of these categories. What you call "moderate contamination" might be called "little contamination" by another student. Just try to be consistent within your evaluation. For **diversity**, look for different colors, shapes, textures, sizes, and anything else that is indicative of a different organism growing, and record the number of different organisms present. **Interpretation** would be relative effectiveness of the agent (excellent, good, fair, poor).

Number on Plate	Hand-washing Agent	"Before"		"After"		Interpretation
		Relative Growth	Diversity	Relative Growth	Diversity	
1						
2						
3						
4						
5						

QUESTIONS

1 *Which agent seemed to be most effective at removing microbes from the fingertips?*

2 *Which agent seemed to be least effective at removing microbes from the fingertips?*

3 *What other variables besides the cleansing agent might be responsible for any differences noted between fingers or students?*

4 *Did you or any other students notice some microbes persisting more than others after washing? What factor(s) might account for this?*

5 *Do some research and fill in the following table. Your answer to "Antibacterial Effect" should address the structural and/or functional part of the cell affected, as well as if the agent is bactericidal (kills bacteria) or bacteriostatic (stops bacterial growth, but doesn't kill them). Under "Other Antimicrobial Effects," note any antiviral, antiprotozoal, and/or antifungal properties of the agent.*

Active Ingredient	Antibacterial Effect	Other Antimicrobial Effects
Triclosan		
Isopropanol/ethanol (isopropyl/ethyl alcohol)		
Benzalkonium chloride		
Povidone iodine scrub		
Chlorhexidine gluconate		

Basic Growth Media

To cultivate microbes, microbiologists use a variety of growth media. Although these media may be formulated from scratch, they more typically are produced by rehydrating commercially available powdered media. Media that are routinely encountered in the microbiology laboratory range from the widely used, general-purpose growth media, to the more specific selective and differential media used in identification of microbes (Sections 4 and 5). In Exercise 1-2 you will learn how to prepare simple general growth media. ◆

Nutrient Broth and Nutrient Agar Preparation

EXERCISE 1-2

Theory

Nutrient broth and nutrient agar are common media used for maintaining bacterial cultures. To be of practical use, they have to meet the diverse nutrient requirements of routinely cultivated bacteria. As such, they are formulated from sources that supply carbon and nitrogen in a variety of forms—amino acids, purines, pyrimidines, monosaccharides to polysaccharides, and various lipids. Generally, these are provided in digests of plant material (phytone) or animal material (peptone and others). Because the exact composition and amounts of carbon and nitrogen in these ingredients are unknown, these media are considered to be **undefined.** They are also known as **complex media**[1].

In most classes (because of limited time), media are prepared by a laboratory technician. Still, it is instructive for novice microbiologists to at least gain exposure to what is involved in media preparation. Your instructor will provide specific instructions on how to execute this exercise using the equipment in your laboratory.

Application

Microbiological growth media are prepared to cultivate microbes. These general growth media are used to maintain bacterial stock cultures.

[1] In contrast, chemically defined media are composed of ingredients with a known chemical structure and in known quantities. For an example, see the recipe for Simmon's Citrate Agar on page 864. In it, the only carbon source is the six-carbon sugar citrate ($C_6H_8O_7$) and the only nitrogen source is ammonium (NH_4^+).

In This Exercise

You will prepare 1-liter batches of two general growth media: nutrient broth and nutrient agar. During the course of the semester, a laboratory technician will probably do this for you, but it is good to gain firsthand appreciation for the work done behind the scenes!

✓ Materials

Per Student Group

- ☐ One 2-liter Erlenmeyer flask for each medium made
- ☐ Three or four 500 mL Erlenmeyer flasks and covers (can be aluminum foil)
- ☐ Stirring hot plate
- ☐ Magnetic stir bars
- ☐ All ingredients listed in the following recipes (or commercially prepared dehydrated media)
- ☐ Sterile Petri dishes
- ☐ Test tubes (16 mm × 150 mm) and caps
- ☐ Balance
- ☐ Weighing paper or boats
- ☐ Spatulas

Medium Recipes

Nutrient Broth

• Beef extract	3.0 g
• Peptone	5.0 g
• Distilled or deionized water	1.0 L

pH 6.6–7.0 at 25°C

1 Nutrient Agar

• Beef extract	3.0 g
• Peptone	5.0 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L

pH 6.6–7.0 at 25°C

Preparation of Medium

Lab One

To minimize contamination while preparing media, clean the work surface, turn off all fans, and close any doors that might allow excessive air movements.

Nutrient Agar Tubes

1. Weigh the ingredients on a balance (Fig. 1.2).
2. Suspend the ingredients in 1 liter of distilled or deionized water in the 2-liter flask, mix well, and boil until fully dissolved (Fig. 1.3).
3. Dispense 7 mL portions into test tubes and cap loosely (Fig. 1.4). If your tubes are smaller than those listed in Materials, adjust the volume to fill 20% to 25% of the tube. Fill to approximately 50% for agar deeps.
4. Sterilize the medium by autoclaving for 15 minutes at 121°C (Fig. 1.5).
5. After autoclaving, cool to room temperature with the tubes in an upright position for agar deep tubes. Cool with the tubes on an angle for agar slants (Fig. 1.6).
6. Incubate the slants and/or deep tubes at 35 ± 2°C for 24 to 48 hours.

Nutrient Agar Plates

1. Weigh the ingredients on a balance (Fig. 1.2).
2. Suspend the ingredients in 1 liter of distilled or deionized water in the 2-liter flask, mix well, and boil until fully dissolved (Fig. 1.3).

1.2 Weighing Medium Ingredients ◆ Solid ingredients are weighed with an analytical balance. A spatula is used to transfer the powder to a tared weighing boat. Shown here is dehydrated nutrient agar, but the weighing process is the same for any powdered ingredient.

1.3 Mixing the Medium ◆ The powder is added to a flask of distilled or deionized water on a hot plate. A magnetic stir bar mixes the medium as it is heated to dissolve the powder.

1.4 Dispensing the Medium into Tubes ◆ An adjustable pump can be used to dispense the appropriate volume (usually 7 mL–10 mL) into tubes. Then, loosely cap the tubes.

3. Divide into three or four 500 mL flasks for pouring. Smaller flasks are easier to handle when pouring plates. Don't forget to add a magnetic stir bar and to cover each flask before autoclaving.
4. Autoclave for 15 minutes at 121°C to sterilize the medium.
5. Remove the sterile agar from the autoclave, and allow it to cool to 50°C while it stirs on a hot plate.
6. Dispense approximately 20 mL into sterile Petri plates (Fig. 1.7). **Be careful! The flask will still be hot, so wear an oven mitt.** While you pour the agar, shield the Petri dish with its lid to reduce the chance of introducing airborne contaminants. If necessary, *gently* swirl each plate so the agar completely covers the bottom; do not swirl the agar up into the lid. Allow the agar to cool and solidify before moving the plates (Fig. 1.8).
7. Store these plates on a countertop for 24 hours to allow them to dry prior to use.

Nutrient Broth

1. Weigh the ingredients on a balance (Fig. 1.2).
2. Suspend the ingredients in 1 liter of distilled or deionized water in the 2-liter flask. Agitate and heat slightly (if necessary) to dissolve them completely (Fig. 1.3).

1.5 Autoclaving the Media ◆ Media are sterilized in an autoclave for 15 minutes at 121°C. Shown are four racks of tubed media being removed from an autoclave. The tubes on the left are brain-heart infusion broth (BHI) and they will be allowed to stand and cool. Once cool, they will remain liquid. The tubes on the right contain brain-heart infusion agar (BHIA), a solid medium, which is liquid when it is removed from the autoclave but will solidify as it cools due to the agar in it. The position of the tube as it cools will determine whether an agar deep tube or an agar slant is produced (Fig. 1.6). Note the angled sides of the racks that allow them to be tipped so the agar will solidify in a slant. Plated media are autoclaved in a flask and then dispensed into sterile Petri dishes (Fig. 1.7).

3. Dispense 7 mL portions into test tubes (or less, depending on your lab customs) and cap loosely (Fig. 1.4). As with agar slants, if your tubes are smaller than those recommended in Materials, add enough broth to fill them approximately 20% to 25%.
4. Sterilize the medium by autoclaving for 15 minutes at 121°C (Fig. 1.5).

1.6 Tubed Media ◆ From left to right: a broth, an agar slant, and an agar deep tube. The solid media are liquid when they are removed from the autoclave. Agar deeps are allowed to cool and solidify in an upright position, whereas agar slants are cooled and solidified on an angle.

1.7 Pouring Agar Plates ◆ Agar plates are made by pouring sterilized medium into sterile Petri dishes. The lid is used as a shield to prevent airborne contamination. Once poured, the dish is gently swirled so the medium covers the base. Plates are then cooled and dried to eliminate condensation.

Lab Two

1 Examine the tubes and plates for evidence of growth.

2 Record your observations on the data sheet, page 25.

1.8 An Agar Plate ♦ Plated media are often used for isolating individual species from a mixed culture (Exercises 1-4 and 1-5) or for counting the number of cells in a diluted sample (Exercise 6-1). Some differential tests also use plated media (e.g., milk agar, DNase agar, starch agar). Shown is a BHIA plate, which stands for "brain-heart infusion agar." The 1.5%–2% agar in the medium acts as a solidifying agent to suspend the nutrients—extracts of brain and heart tissues. Brain and heart provide carbon, nitrogen, and other essential nutrients for growth, as well as energy.

Reference

Zimbro, Mary Jo and David A. Power. Pages 404–405 and 408 in *DIFCO™ & BBL™ Manual—Manual of Microbiological Culture Media.* Sparks, MD: Becton, Dickinson and Company, 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 1-2

Nutrient Broth and Nutrient Agar Preparations

OBSERVATIONS AND INTERPRETATIONS

1 In the following table, record the number of each medium type you prepared, and then record the number of apparently sterile ones. Calculate your percentage of successful preparations for each. In the last column, speculate as to probable/possible sources of contamination (even if all your media are sterile).

Medium	Total Number Prepared	Number of Sterile Preparations	Percentage of Successful Preparations	Probable Sources of Contamination
Nutrient agar tubes (slant or deep)				
Nutrient agar plates				
Nutrient broths				

QUESTIONS

1 *Which medium was most difficult to prepare without contamination? Why do think this might be so?*

1

2 *For each of the following types of contamination suggest the most likely point in preparation (or later) at which the contaminant was introduced.*

a. *Growth in all broth tubes.*

b. *Growth in one broth tube.*

c. *Growth only on the surface of a plate.*

d. *Growth throughout the agar's thickness on a plate.*

e. *Growth only in the upper 1 cm of agar in an agar deep tube.*

f. *All plates in a batch have the same type and density of contaminants.*

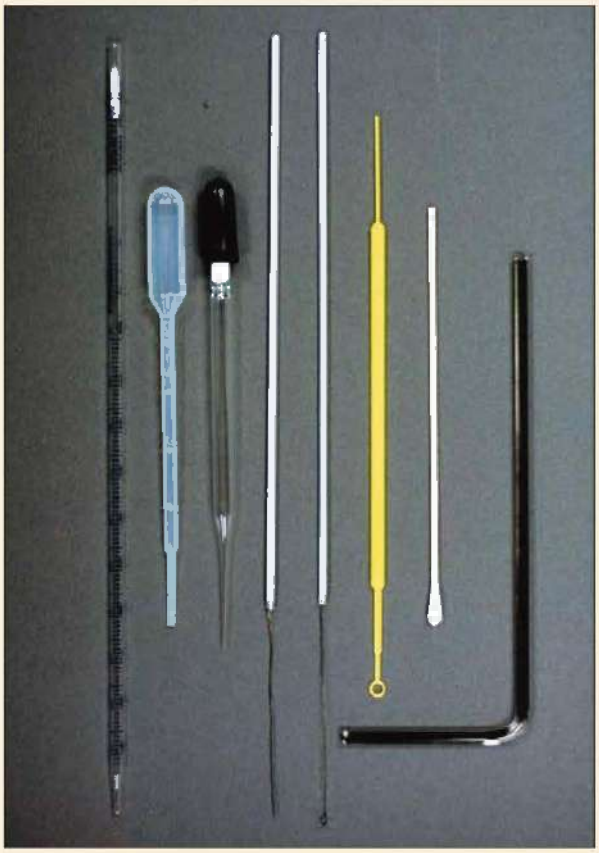
g. *Only a few plates in a batch are contaminated, and each looks different.*

Aseptic Transfers and Inoculation Methods

As a microbiology student, you will be required to transfer living microbes from one place to another aseptically (i.e., without contamination of the culture, the sterile medium, or the surroundings). While you won't be expected to master all transfer methods right now, you will be expected to perform most of them over the course of the semester. Refer to Exercises 1-3 through 1-5 and Appendices B, C, and D as needed.

To prevent contamination of the sample, inoculating instruments (Fig. 1.9) must be sterilized prior to use. Wire inoculating loops and needles are sterilized immediately before use in an incinerator or Bunsen burner flame. The mouths of tubes or flasks containing cultures or media are also incinerated at the time of transfer by passing their openings through a flame. Instruments that are not conveniently or safely incinerated, such as Pasteur pipettes, cotton applicators, glass pipettes, and digital pipettor tips, are sterilized inside wrappers or containers by autoclaving prior to use.

Aseptic transfers are not difficult; however, a little preparation will help assure a safe and successful procedure. Before you begin, you will need to know where the sample is coming from, its destination, and the type of transfer instrument to be used. These exercises provide step-by-step descriptions of routine transfer methods. Certain less-routine transfer methods are discussed in Appendices B through D. ◆



1.9 Inoculating Instruments ◆ Any of several different instruments may be used to transfer a microbial sample, the choice of which depends on the sample source, its destination, and any special requirements imposed by the specific protocol. Shown here are several examples of transfer instruments. From left to right: serological pipette (see Appendix C), disposable transfer pipette, Pasteur pipette, inoculating needle, inoculating loop, disposable inoculating needle/loop, cotton swab (see Appendix B and Exercise 1-4), and glass spreading rod (see Exercise 1-5). (***Note:*** the glass rod is not an inoculation instrument, but it is used to spread an inoculum introduced to an agar plate by another instrument. As such, it is an instrument used in an inoculation process.) When transferring BSL-2 organisms, we advise using a sterile disposable loop or wooden stick (not shown). Neither of these requires incineration after use and minimize the threat of aerosol production.

Common Aseptic Transfers and Inoculation Methods

EXERCISE 1-3

Theory

A medium that contains living microbes is called a **culture.** If a culture contains a single species it is said to be a **pure culture.** It is essential to transfer microbes from their pure culture to a sterile medium **aseptically,** that is, without contamination of yourself, others, the environment, the source culture, or the medium being inoculated. In other words, you want your pure culture to stay pure, your new culture to be pure, and the surroundings to remain uninoculated.

Following are general techniques and practices that improve your chances of successfully making an aseptic transfer. Equally important are the techniques related to safety. It has been demonstrated that while "...the causative incident for most LAIs (laboratory acquired infections) is unknown...A procedure's potential to release microorganisms into the air as aerosols and droplets is the most important operational risk factor..." (Chosewood and Wilson, 2009). In other words, limiting aerosol production is a *safety issue and not an issue of keeping pure cultures pure.* Please adhere to these practices for your safety and the safety of those around you.

- **Minimize the potential of contamination.** Do not perform any transfers over your books and papers because you may inadvertently and unknowingly contaminate them with droplets or aerosols that settle. Put them safely away. Some labs advise performing transfers over a disinfectant-soaked paper towel.
- **Be organized.** Arrange all media in advance and clearly label them with your name, the date, the medium, and the inoculum (Fig. 1.10). Tubes may be labeled with tape, paper held on with a rubber band, or by writing directly on the glass (this option presumes you will clean the writing off before you dispose of the culture for decontamination). You *should* write directly on the base (not the lid) of plastic Petri plates because they are disposable. Be sure not to place any labels in such a way as to obscure or obstruct your view of the tube's or plate's interior.
- **Place all media tubes in a test tube rack when not in use whether they are sterile or not.** Tubes should never be laid on the table surface because they may leak (Fig. 1.11).
- **Take your time.** Work efficiently, but do not hurry. You are handling potentially dangerous microbes. Working at a frenzied pace leads to carelessness and accidents.
- **Never hold a tube culture by its cap.** Caps are generally loose to allow aeration and are not secure enough to be used as a handle. Even screw caps can be loose enough so as not to be secure.
- **Hold the inoculating loop or needle like a pencil in your dominant hand and relax** (Fig. 1.12)!
- **Adjust your Bunsen burner so its flame has an inner and outer cone** (Fig. 1.13).

1.10 Label The Media ◆ To avoid confusion after-the-fact, it is best to label sterile media prior to inoculating it. (A) Tubed media can be labeled with tape, paper labels, or directly on the glass with a marking pen. Labels must be removed when tubes are put in the autoclave bin for sterilization. (B) Plastic Petri dishes should be labeled on their base, not on their lid, because the lid may get separated from its base during reading or rotated from its correct orientation. Because most labs use disposable Petri dishes, the labels do not have to be removed prior to autoclaving.

1.11 Microbiologist at Work ◆ Materials are neatly positioned and not in the way. To prevent spills, culture tubes are stored upright in a test tube rack. They are never laid on the table. The microbiologist is relaxed and ready for work. Notice he is holding the loop like a pencil, not gripping it like a dagger.

1.12 Hold the Loop Like a Pencil ◆ Holding the loop as shown puts the hand in a convenient position to hold tube caps by the "pinky" finger.

1.13 Bunsen Burner Flame ◆ When properly adjusted, a Bunsen burner produces a flame with two cones. Sterilization of inoculating instruments is done in the hottest part of the flame—the tip of the inner cone (red arrow). Heat-fixing bacterial smears on slides and incinerating the mouths of open glassware items may be done in the outer cone (white arrow).

Application

Media come in many forms, each with specific applications. **Broths** are used to grow microbes when fresh cultures or large numbers of cells are required. Broths of differential media are also used in microbial identification (Section 5). **Agar slants** are generally used to grow stock cultures that can be refrigerated after incubation and maintained for several weeks. In addition, many differential media are agar slants. **Plated media** are typically used for obtaining isolation of species (Exercises 1-4 and 1-5), differential testing, and quantifying bacterial densities (Exercise 6-1). In all cases, using these media requires aseptic inoculation in which a portion of an existing pure culture is transferred to a sterile medium to start a new pure culture. (A streak plate and sometimes a spread plate are exceptions to this. See Exercises 1-4 and 1-5.)

Transfers can be made between all forms of media—slants, broths, and plates—depending on the intended use of the new culture. The following is organized into transfers from broth culture to sterile broth, agar slant culture to sterile agar slant, and plate culture to sterile broth. If you can do these, then you have the skills to transfer between most any combination of media. (Inoculation of sterile agar plates is covered in Exercises 1-4 and 1-5 and will complete your skill set.)

The instruments usually used for transfers are either **inoculating loops** or **inoculating needles.** (Pipettes are also sometimes used and these are covered in Appendices C and D.) For simplicity, the following instructions only refer to inoculating loops, but the same apply to inoculating needles.

A Special Note about Transferring BSL-2 Organisms

Most college biology teaching laboratories have eliminated or reduced the use of BSL-2 organisms, as we have in this edition of the lab manual. However, use of some BSL-2 organisms is unavoidable for some tests. In other exercises, they are included as optional test organisms.

The primary concern with BSL-2 organisms is contamination or infection due to inhalation of aerosols. Aerosols are problematic because we generally are unaware of their production and they remain suspended in the air long after the procedure has been completed. Throughout this section we emphasize techniques that minimize aerosol production, but they are even more essential when handling BSL-2 organisms. Your lab will have specific guidelines on how to handle BSL-2 organisms and you should take these seriously. They may include any or all of the following precautions, depending on the exercise and the equipment you have available.

- Performing tests in a Class II biosafety cabinet (Fig. I.1) is recommended, but not all teaching laboratories have these installed.
- Using an electric incinerator (Fig. 1.14) for decontaminating wire loops and needles, which simultaneously contains aerosols within the ceramic interior and decontaminates them.
- Using sterile disposable loops/needles or wooden sticks that don't require flaming after use and can be disposed of in sharps containers or other appropriate receptacles for autoclaving.

1.14 Bacteriological Incinerator ◆ Bacterial incinerators use infrared heat and reach temperatures over 800°C. A wire loop/needle is inserted into the incinerator and heated until the wire is red-hot (usually within 5–7 seconds). (The handle may also get hot, so be careful.) The loop/needle is then removed and allowed to cool without touching anything. It may then be used to transfer microbes, or if this is done at the completion of a transfer, it may be set aside until needed again.

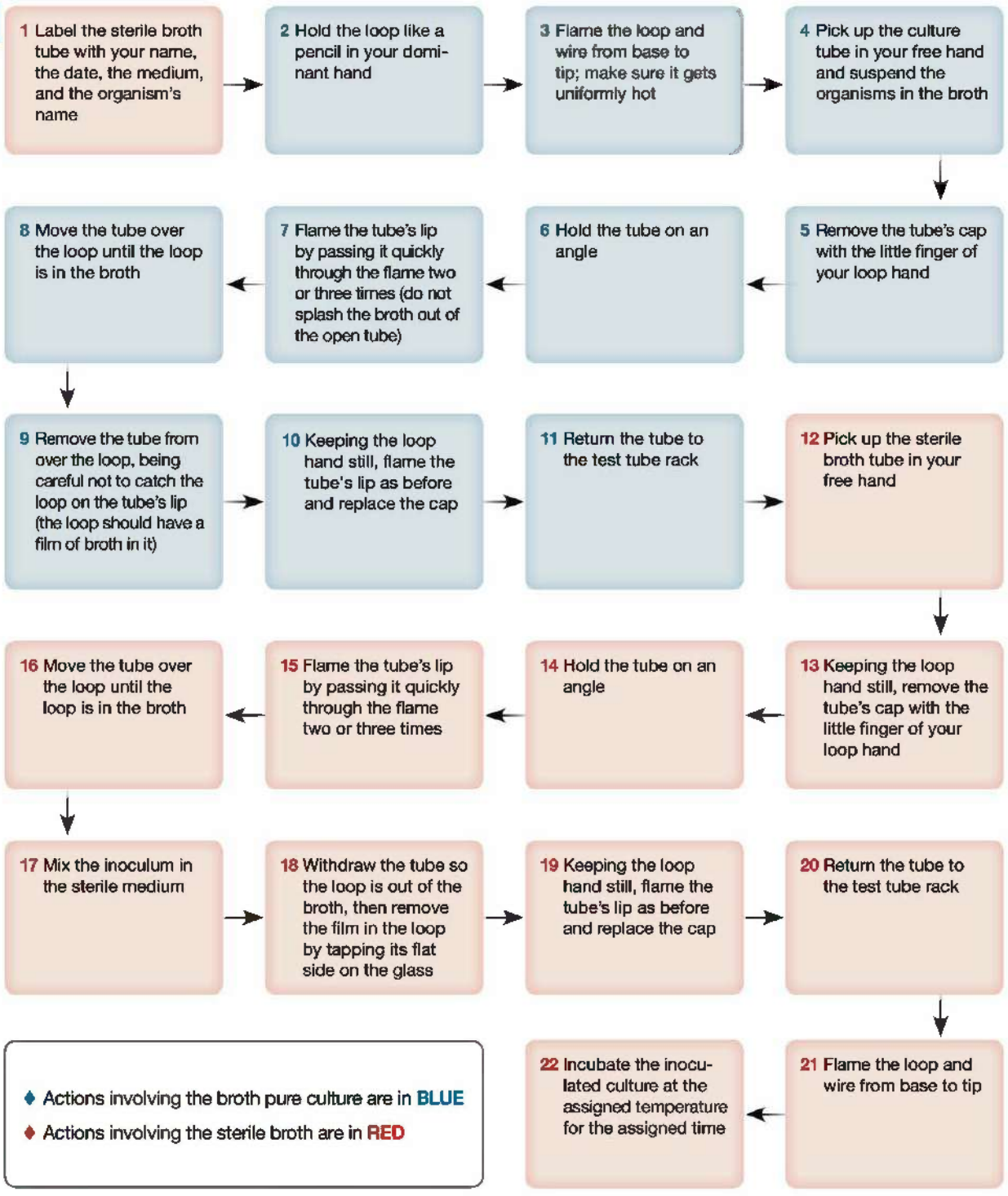
- Wearing gloves and eye protection are also recommended.
- Identifying BSL-2 organisms with red caps on tube cultures or BSL-2 labels on plate cultures.

Whatever precautions your college's guidelines dictate, take them seriously. Your health and the health of others in your lab are at stake.

Transfer from a Broth Culture to a Sterile Broth

As you read these instructions, also follow the procedural diagram in Figure 1.15 to get a summary view of the process. Make appropriate adjustments if handling a BSL-2 organism.

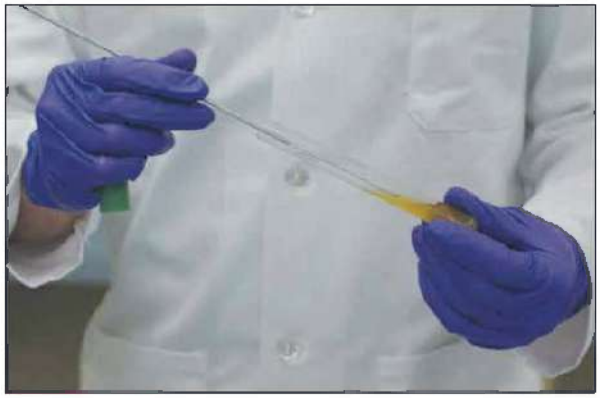
1. Label the sterile broth tube with your name, the date, and the organism you are inoculating it with.
2. Make sure your loop is a closed circle. If it isn't, pinch it closed. Hold it like a pencil, and then flame it from base to tip as shown in Figure 1.16. Be sure the entire wire reaches orange-hot at some point.
3. Suspend the bacteria in the broth culture with a vortex mixer prior to transfer (Fig. 1.17). Be sure not to mix so vigorously that broth gets into the cap or that you lose control of the tube. Start slowly, and then gently increase the speed until the tip of the vortex reaches the bottom of the tube. Alternatively, the culture may be agitated by drumming your fingers along the length of the tube several times (Fig. 1.18). Again, be careful not to splash broth into the cap or lose control of the tube.
4. Loosen the cap of the culture tube (this is especially important if you are using a screw-cap tube). Move the culture tube to your loop hand. Remove and hold the tube's cap with the little finger of your loop hand (Fig. 1.19). Moving the tube and not the loop prevents excessive movement of the loop that might result in aerosols or droplets.
5. Hold the tube on an angle to minimize the opportunity for airborne contamination (Fig. 1.20).
6. Incinerate the lip of the tube by passing it quickly through the flame two or three times (Fig. 1.21). Do not wave it through the flame so fast that broth sloshes out of the open end.


1 Label the sterile broth tube with your name, the date, the medium, and the organism's name
2 Hold the loop like a pencil in your dominant hand
3 Flame the loop and wire from base to tip; make sure it gets uniformly hot
4 Pick up the culture tube in your free hand and suspend the organisms in the broth
5 Remove the tube's cap with the little finger of your loop hand
6 Hold the tube on an angle
7 Flame the tube's lip by passing it quickly through the flame two or three times (do not splash the broth out of the open tube)
8 Move the tube over the loop until the loop is in the broth
9 Remove the tube from over the loop, being careful not to catch the loop on the tube's lip (the loop should have a film of broth in it)
10 Keeping the loop hand still, flame the tube's lip as before and replace the cap
11 Return the tube to the test tube rack
12 Pick up the sterile broth tube in your free hand
13 Keeping the loop hand still, remove the tube's cap with the little finger of your loop hand
14 Hold the tube on an angle
15 Flame the tube's lip by passing it quickly through the flame two or three times
16 Move the tube over the loop until the loop is in the broth
17 Mix the inoculum in the sterile medium
18 Withdraw the tube so the loop is out of the broth, then remove the film in the loop by tapping its flat side on the glass
19 Keeping the loop hand still, flame the tube's lip as before and replace the cap
20 Return the tube to the test tube rack
21 Flame the loop and wire from base to tip
22 Incubate the inoculated culture at the assigned temperature for the assigned time
♦ Actions involving the broth pure culture are in BLUE
♦ Actions involving the sterile broth are in RED

1.15 Procedural Diagram for an Aseptic Transfer from a Nutrient Broth Pure Culture to a Sterile Nutrient Broth Tube ♦ This is a summary of the procedure. Make every effort to keep your loop hand as still as possible throughout the transfer. Details can be found in the text. Make appropriate adjustments if transferring a BSL-2 organism.

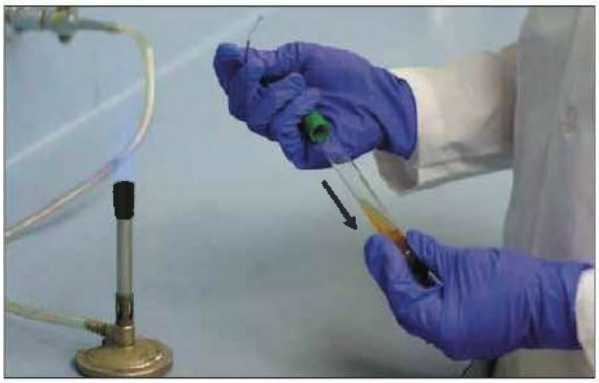
1.16 Flaming Loop ♦ Incineration of an inoculating loop's wire is done by passing it through the tip of the flame's inner cone. Begin at the wire's base and continue to the end, making sure that all parts are heated to a uniform orange color. Allow the wire to cool before touching it or placing it on/in a culture. The former will burn you; the latter will cause aerosols of microorganisms.

1.18 Mixing Broth by Hand ♦ A broth culture always should be mixed prior to transfer. Tapping the tube with your fingers gets the job done safely and without special equipment.



1.20 Holding the Tube at an Angle ♦ The tube is held at an angle to minimize the chance that airborne microbes will drop into it. Notice that the tube's cap is held in the loop hand.

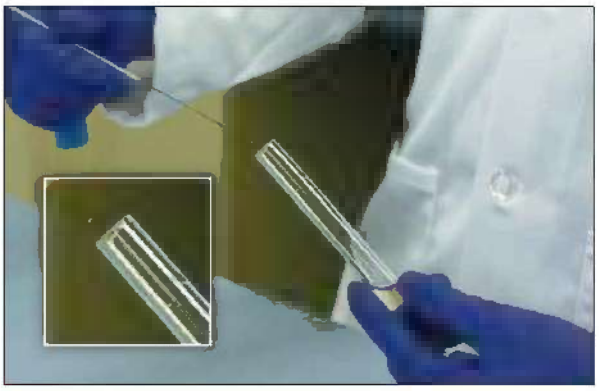
1.17 Vortex Mixer ♦ Bacteria may be suspended in a broth using a vortex mixer. The switch on the bottom has three positions: "auto" (left), "off" (center), and "on" (right). The rubber boot is activated when touched only if the "auto" position is used; "on" means the boot is constantly vibrating. Above the on/off/auto switch is a variable speed knob. The slowest speed that allows the vortex to reach the bottom of the tube is used. Caution must be used to prevent broth from getting into the cap or losing control of the tube and causing a spill (note the hand position around the tube, ready to grab it) Short bursts of vortexing can be used if the glassware is too full to allow vortexing to the bottom.



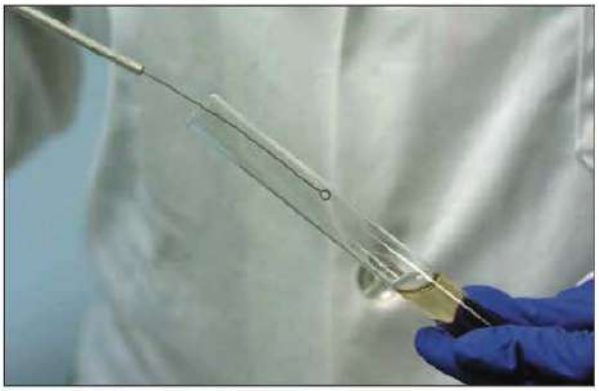
1.19 Removing the Tube Cap ♦ The loop is held in the dominant hand and the tube in the other hand. Remove the tube's cap with the little finger of your loop hand by pulling the tube away with the other hand; keep your loop hand still. Hold the cap in your little finger during the transfer. When replacing the cap, move the tube back to the cap to keep your loop hand still. The replaced cap doesn't have to be on firmly at this time—just enough to cover the tube.

1.21 Flaming the Tube ♦ The tube's mouth is passed quickly through the flame a couple of times to sterilize the tube's lip and the surrounding air. Notice that the tube's cap is held in the loop hand.

1.22 Move the Tube, Not the Loop ◆ The open tube is held at an angle to minimize airborne contamination of it. When placing a loop into a broth tube or removing it, keep the loop hand still and move the tube. ***Be careful not to catch the loop on the tube's lip when removing it.*** This produces aerosols that can be dangerous or produce contamination.



1.23 Do Not Catch the Loop on the Tube's Lip When Removing It ◆ Notice the film of broth in the loop (see inset). Be careful not to catch the loop on the lip of the tube when removing it. This would produce aerosols and droplets that can be dangerous or produce contamination.



1.24 Removing Excess Broth from Loop after Transferring ◆ Before removing it from the new culture tube, tap the face of the loop on the glass to remove the broth film (Fig. 1.23). Failing to do so will result in splattering and aerosols when sterilizing the loop in a flame.

7 This step is important to minimize the production of aerosols. Hold the loop hand still and move the tube up the wire until the loop's tip is in the broth. Continue holding the loop hand still while you remove the tube from over the loop (Fig. 1.22). *Be careful not to catch the loop on the lip of the tube or you will produce contaminated droplets and aerosols.* At this point, there should be a visible film of broth in your loop (Fig. 1.23). If there isn't a visible film of broth, replace the cap, flame the loop, let it cool, and then pinch the loop so it is a closed circle. Then, start over.

8 While holding your loop hand still, flame the tube and replace the cap. Set the tube in the rack and pick up the sterile broth tube in your free hand.

9 Repeat the process of flaming the lip of the sterile broth tube while holding the loop hand still. Don't forget you have living microbes exposed to the environment on the loop at this point and careless movements can spread them.

10 Insert the loop into the sterile broth and mix by gently swirling the loop.

11 Before you remove the loop from the tube, tap the loop's face on the inside of the tube to remove the film within the loop (Fig. 1.24). Be persistent in this—don't give up until you are successful at its removal. If you were to flame the loop with the film present, you would produce aerosols.

12 Keeping your loop hand still, flame the tube, replace the cap, and set it in the rack.

13 Flame the loop from base to tip until it is uniformly orange-hot.

Transfer from Agar Slant Culture to Sterile Agar Slant

As you read these instructions, also follow the procedural diagram in Figure 1.25 to get a summary view of the process. This transfer has a lot in common with the broth-to-broth transfer and you are referred back to it at relevant points. **Make appropriate adjustments if handling a BSL-2 organism.**

1 Label the sterile agar slant with your name, the date, the medium, and the inoculum.

2 Hold the loop like a pencil and then flame it from base to tip as in a broth transfer (Fig. 1.16).

3 Loosen and remove the cap on the culture tube as in a broth transfer (Fig. 1.19).

4 Hold the culture tube on an angle with the agar surface facing upward.

5 Flame the tube's lip as in a broth transfer (Fig. 1.21).

1 Label the sterile agar slant with your name, the date, the medium, and the organism's name
2 Hold the loop like a pencil in your dominant hand
3 Flame the loop and wire from base to tip, making sure it gets uniformly orange-hot
4 Pick up the culture slant in your free hand
5 Remove the culture tube's cap with the little finger of your loop hand
6 Hold the tube on an angle with the agar surface facing upward
7 Flame the tube's lip by passing it quickly through the flame two or three times
8 Move the tube up the loop's wire until the loop is over some growth
9 Touch the sterile loop to the growth on the agar surface and pick up a small amount of growth
10 Remove the tube from over the loop, being careful not to catch the loop on the tube's lip
11 Keeping the loop hand still, flame the tube's lip as before, and replace the cap
12 Return the culture slant to the test tube rack
13 Pick up the sterile agar slant in your free hand
14 Remove the tube's cap with the little finger of your loop hand
15 Hold the tube on an angle with the agar surface facing upward
16 Flame the tube's lip by passing it quickly through the flame two or three times
17 Move the tube upward over the wire so the loop is at the base of the agar
18 Touch the agar with the tip of the loop
19 Slowly remove the tube from over the loop as you move the loop in a zigzag pattern on the agar surface (do not cut the agar)
20 Remove the tube completely from over the loop without catching the loop on the tube's lip
21 Keeping the loop hand still, flame the tube's lip as before and replace the cap
22 Return the inoculated tube to the test tube rack
23 Flame the loop and wire from base to tip, making sure it gets uniformly orange-hot
24 Incubate the inoculated culture at the assigned temperature for the assigned time
♦ Actions involving the agar slant pure culture are in BLUE
♦ Actions involving the sterile agar slant are in RED

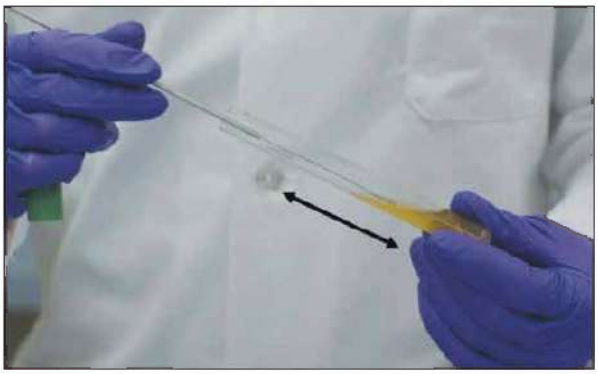
1.25 Procedural Diagram for an Aseptic Transfer from a Nutrient Agar Slant Pure Culture to a Sterile Nutrient Agar Slant ♦
This is a summary of the procedure. Make every effort to keep your loop hand as still as possible throughout the transfer. Details can be found in the text. Make appropriate adjustments if transferring a BSL-2 organism.

6 Move the culture tube up the wire of the loop, and then gently touch the loop's tip to the growth on the agar's surface (Fig. 1.26). You don't need to dig into the agar, nor do you need to scoop up a glob of growth. Just touch the loop to the growth and pick up the smallest amount you can see with your naked eye.

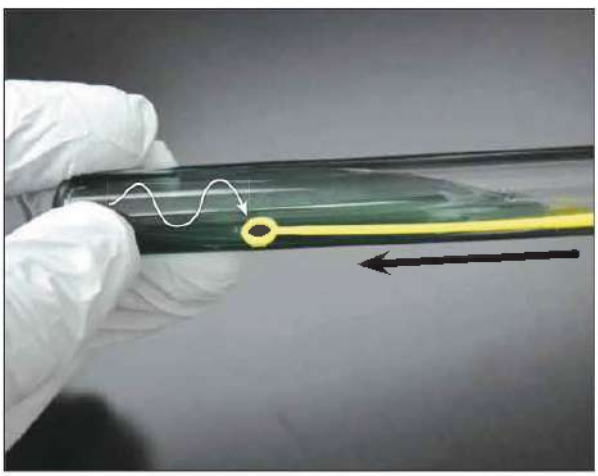
7 Holding your loop hand still, carefully remove the tube from over the wire, flame the culture tube's lip, and replace its cap. Place the culture in the test tube rack.

8 Pick up the sterile agar slant, remove the cap, and flame the tube as before.

9 Move the tube over the wire so the loop is near the bottom of the slant.



1.26 A Loop and an Agar Slant ♦ When placing a loop into a slant tube or removing it, the loop hand is kept still while the tube is moved. Hold the tube so the agar is facing upward. To pick up the inoculum, you only need to gently touch the growth on the agar surface.



1.27 Fishtail Inoculation of a Slant ♦ Begin at the base of the slant and gently move the loop back and forth as you withdraw the tube. Be careful not to cut the agar. After completing the transfer, sterilize the loop or dispose of it properly.

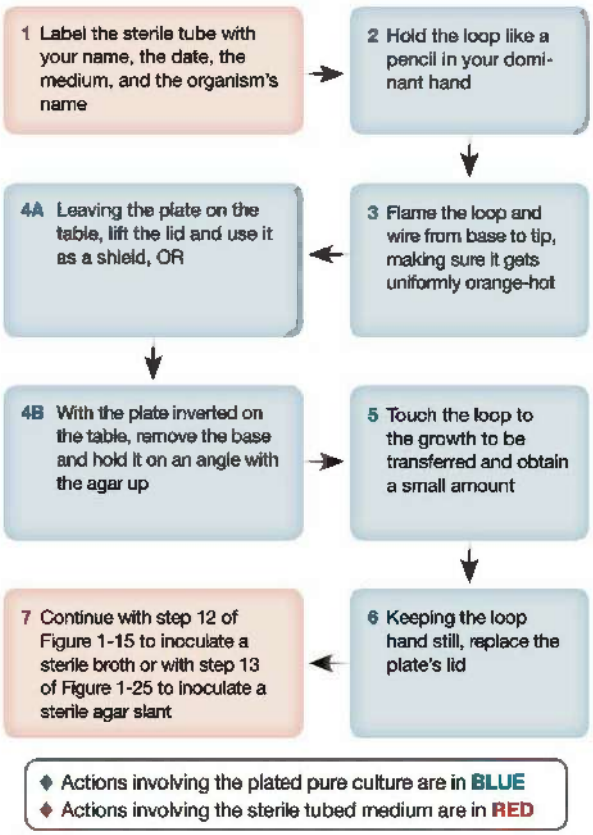
10 Touch the tip of the loop to the agar where there is organism. Then, as you withdraw the tube, move the loop back and forth (Fig. 1.27). Be careful not to cut the agar with the loop. This is called a **fishtail inoculation** or **fishtail streak** because you are seeding the agar surface in a wavy pattern resembling the movement of a fish tail.

11 Be careful not to catch the loop on the tube's lip as you remove it. Then, keeping the loop hand still, flame the tube's lip, replace its cap, and put it in the test tube rack.

12 Flame the loop from base to tip as before.

Transfers from Plate Culture to Sterile Broth

As you read these instructions, also follow the procedural diagram in Figure 1-28 to get a summary view of the process. Make appropriate adjustments if transferring a BSL-2 organism.


1 Label the sterile tube with your name, the date, the medium, and the organism's name
2 Hold the loop like a pencil in your dominant hand
3 Flame the loop and wire from base to tip, making sure it gets uniformly orange-hot
4A Leaving the plate on the table, lift the lid and use it as a shield, OR
4B With the plate inverted on the table, remove the base and hold it on an angle with the agar up
5 Touch the loop to the growth to be transferred and obtain a small amount
6 Keeping the loop hand still, replace the plate's lid
7 Continue with step 12 of Figure 1-15 to inoculate a sterile broth or with step 13 of Figure 1-25 to inoculate a sterile agar slant
♦ Actions involving the plated pure culture are in BLUE
♦ Actions involving the sterile tubed medium are in RED

1.28 Procedural Diagram for an Aseptic Transfer from a Nutrient Agar Plate Pure Culture to a Sterile Tubed Medium ♦ This is a summary of the procedure. Make every effort to keep your loop hand as still as possible throughout the transfer. Details can be found in the text. Inoculation of a sterile broth is the same as in Figure 1.15, whereas inoculation of a sterile slant is the same as in Figure 1.25. Make appropriate adjustments if transferring a BSL-2 organism.

1. Flame the loop from base to tip (Fig. 1.16).
2. Lift the lid of the Petri dish and use it as a shield from airborne contamination (Fig. 1.29).
3. Touch the loop to an uninoculated portion of the plate to cool it. (Loop wires can get very hot if a series of transfers are made in a short period of time. Placing a hot wire on growth may cause the growth to spatter and create aerosols. If you aren't doing a lot of successive transfers, this is probably unnecessary.) Gently touch the loop to some growth on the agar surface and collect the smallest amount you can see. As with the slant, you don't need to dig into the agar or scoop up a glob of growth.
4. Remove the loop and replace the lid.
5. Pick up the sterile broth and continue with step 9 in the broth-to-broth transfer (page 34 and Fig. 1.15).

In This Exercise

You now have the procedures for obtaining growth from a broth culture, an agar slant culture, and a plated culture. You also have the procedures for inoculating a sterile broth and a sterile agar slant. These can be performed in any combination necessary. Today, you will begin by testing your baseline dexterity by using sterile agar and sterile broth as the "culture" tubes and the tubes to be inoculated. After a little practice with these "blanks," you will do transfers with real cultures and sterile media in the following combinations: agar slant culture to sterile agar slant and sterile broth, broth culture to sterile agar slant and sterile broth, and plate culture to sterile broth (Fig. 1.30).

1.29 "Picking" a Colony for Transfer ♦ Touch the tip of the loop to an isolated colony and get a small amount of growth. Use the lid as a shield from airborne contamination.

✓ Materials

Per Student

- ☐ Inoculating loop
- ☐ Bunsen burner
- ☐ Four sterile nutrient broth tubes
- ☐ Three sterile nutrient agar slants
- ☐ Marking pens and labeling tape (or materials for the preferred labeling method in your laboratory)
- ☐ (Optional) vortex mixer

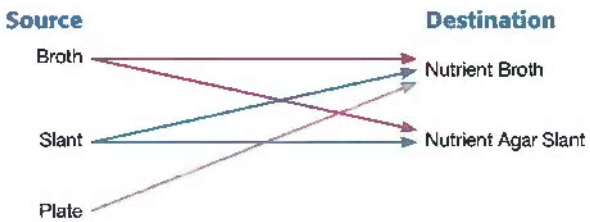
Per Student Group

- ☐ Nutrient agar (NA) slant culture of *Staphylococcus epidermidis*
- ☐ Nutrient broth (NB) culture of *Staphylococcus epidermidis*
- ☐ Nutrient agar plate of *Staphylococcus epidermidis*

Procedure

Lab One

1. Label one nutrient broth (NB) and one nutrient agar (NA) tube with your name and the word "sterile."
2. Using the sterile NB tube and the sterile NA tube, practice making transfers between them in all possible combinations. Your lab partner has the same assignment using a second set of tubes. Work with her/him and alternate transferring and evaluating each other's technique. (Not only will you learn by doing and evaluating another person, alternating will allow the tubes to cool.)
3. Incubate the sterile tubes at 35 ± 2°C until the next lab period.



1.30 Procedural Diagram for Making Transfers of *S. epidermidis* ♦ In today's lab, you will make transfers from broth and slant cultures of *S. epidermidis* to sterile nutrient broth and nutrient agar slant media. You will also transfer from a plate culture of *S. epidermidis* to a nutrient broth.

4 Once you have been "cleared" to make transfers using real cultures, perform the following:

a Label a sterile nutrient agar slant and a sterile nutrient broth with your name, the medium in the tube (either NA or NB), the source of inoculum (NA slant), and the organism. Then, aseptically transfer from the *S. epidermidis* NA slant culture to the sterile NA slant and the sterile NB.

b Label a sterile NA slant and a sterile NB with your name, the medium in the tube, the source of inoculum (NB), and the organism. Then, transfer from the *S. epidermidis* NB to the sterile NA slant and the sterile NB.

c Label a sterile NB with your name, the medium in the tube, the source of inoculum (NA plate), and the organism. Then, transfer from the *S. epidermidis* NA plate culture to the sterile NB. Choose a well-isolated colony and touch the center with the loop as in Figure 1.29.

d Incubate these five tubes at 35 ± 2°C until the next lab period.

Lab Two

1 Remove your cultures and "sterile" practice tubes from the incubators and examine them for growth. Record your observations and answer the questions on the data sheet, page 39.

2 Your instructor may ask you to save your cultures for later use. If so, put them in the refrigerator. Otherwise, remove the labels and dispose of them in the appropriate autoclave container.

References

Barkley, W. Emmett and John H. Richardson. Chap. 29 in *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology, 1994.

Chosewood, L. Casey and Deborah E. Wilson, eds. Page 4 in *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Department of Health and Human Services Publication No. (CDC) 21-1112, December 2009.

Claus, G. William. Chap. 2 in *Understanding Microbes—A Laboratory Textbook for Microbiology*. New York: W. H. Freeman and Company,1989.

Darlow, H. M. Chap. VI in *Methods in Microbiology*, Vol. 1. J. R. Norris and D. W. Ribbins, eds. London, UK: Academic Press, Ltd., 1969.

Emmert, Elizabeth A. B., Jeffrey Byrd, Ruth A. Gyure, Diane Hartman, and Amy White et al. "Biosafety Guidelines for handling Microorganisms in the Teaching Laboratory: Development and Rationale." *Journal of Microbiology & Biology Education* (May 2013): 78–83. DOI: http://dx.doi.org/10.1128/jmbe.v14i1.531.

Estridge, Barbara and Anna Reynolds. Page 712 in *Basic Clinical Laboratory Techniques*, 6th ed. Independence, KY: Cengage, 2012.

Fleming, Diane O. Chap. 13 in *Laboratory Safety—Principles and Practices*, 2nd ed. Diane O. Fleming, John H. Richardson, Jerry J. Tulis, and Donald Vesley, eds. Washington, DC: American Society for Microbiology, 1995.

Koneman, Elmer W., Stephen D. Allen, William M. Janda, Paul C. Schreckenberger, and Washington C. Winn, Jr. Chap. 2 in *Color Atlas and Textbook of Diagnostic Microbiology*, 5th ed. Philadelphia: Lippincott-Raven Publishers, 1997.

Murray, Patrick R., Ellen Jo Baron, Michael A. Pfaller, Fred C. Tenover, and Robert H. Yolken. *Manual of Clinical Microbiology*, 6th ed. Washington, DC: American Society for Microbiology, 1995.

Power, David A. and Peggy J. McCuen. *Manual of BBL™ Products and Laboratory Procedures*, 6th ed. Cockeysville, MD: Becton Dickinson Microbiology Systems, 1988.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 1-3

Common Aseptic Transfers and Inoculation Methods

OBSERVATIONS AND INTERPRETATIONS

1 Describe the appearance of growth on/in each medium. Growth on a solid medium could be described by color and amount (abundant, sparse, absent). Draw representative samples of each growth type. Growth in broth can be described by its degree of cloudiness (turbidity), using a qualitative scoring system:

+++ means "very turbid"
++ means "somewhat turbid"
+ means "barely turbid"
0 means "not turbid"

Source	Medium Inoculated	
	Nutrient Broth	Nutrient Agar Slant
"Sterile" practice media		
Staphylococcus epidermidis on NA slant		
Staphylococcus epidermidis in NB		
Staphylococcus epidermidis on sterile NA Plate		NA

QUESTIONS

1 *Which medium was most difficult for you to transfer from? Which medium was most difficult for you to inoculate? Explain your difficulties.* (**Note:** *There are not correct answers to these questions. They are based on evaluation of your personal experience.*)

2 *Did you get growth on/in the sterile NB and NA slant tubes you practiced with? If not, congratulations. If so, where did you see it and what might have been its source(s)?*

3 *Did you notice a difference in density (turbidity) of growth in NB tubes inoculated from NB and NA slants? Suggest possible reasons why a difference might occur.*

4 *Did you notice a difference in density of growth on NA slants inoculated from NA slants and NB? Suggest possible reasons why a difference might occur.*

Streak Plate Methods of Isolation

Theory

A microbial culture consisting of two or more species is said to be a **mixed culture**, whereas a **pure culture** contains only a single species. Obtaining isolation of individual species from a mixed sample is generally the first step in identifying an organism. A commonly used **isolation technique** is the **streak plate** (Fig. 1.31).

In the streak plate method of isolation, a bacterial sample (always assumed to be a mixed culture) is streaked over the surface of a plated agar medium. During streaking, the cell density decreases, eventually leading to individual cells being deposited separately on the agar surface. Cells that have been sufficiently isolated will grow into **colonies** consisting only of the original cell type. Because some colonies form from individual cells and others from pairs, chains, or clusters of cells, the term **colony-forming unit (CFU)** is a more correct description of the colony origin.

Several patterns are used in streaking an agar plate, the choice of which depends on the source of inoculum and microbiologist's preference. Although streak patterns range from simple to more complex, all are designed to separate deposited cells (CFUs) on the agar surface so individual cells (CFUs) grow into isolated colonies. A quadrant streak or a T-streak is generally used with samples suspected of high cell density, whereas a simple zigzag (continuous streak) pattern may be used for samples containing lower cell densities.

1.31 Quadrant Streak Plate of *Serratia marcescens* ◆
Note the decreasing density of growth in the four streak patterns. On this plate, isolation is first achieved in the second streak, but the microbiologist wouldn't know that at the time of streaking, so all four streaks are performed in the hope that isolation will occur in at least one of them. Cells from an isolated colony (one that's not touching another colony) can be transferred to a sterile medium to start a pure culture.

Application

The identification process of an unknown microbe relies on obtaining a pure culture of that organism. The streak plate method produces individual colonies on an agar plate. A portion of an isolated colony then may be transferred to a sterile medium to start a pure culture.

Following are descriptions of streak techniques.

Inoculation of Agar Plates Using the Quadrant Streak Method

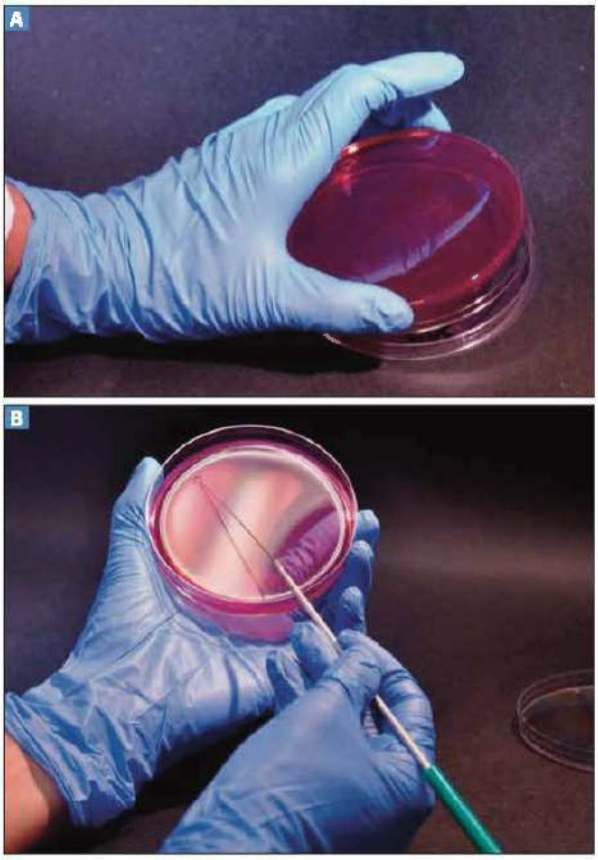
This inoculation pattern is usually performed as the initial streak for isolation of two or more bacterial species in a mixed culture with suspected high cell density.

1. Obtain the sample of mixed culture with a sterile loop.
2. You have two options at this point. Use whichever is more comfortable for you or is required by your instructor.
   a. Leave the sterile agar plate on the table and lift the lid slightly, using it as a shield from airborne contamination (Fig. 1.32).

   or

   b. Place the plate lid down on the table (Fig. 1.33A). Then remove the base and hold it in the air on an angle (Fig. 1.33B).
3. Starting at the edge of the plate lightly drag the loop back and forth across the agar surface as shown in Figure 1.34A. Be careful not to cut the agar surface. The loop should contact the agar as shown in Figure 1.35.
4. Remove the loop and replace the lid.
5. Sterilize your loop as before. It is especially important to flame it from base to tip now because the loop has bacteria on it.
6. Rotate the plate a little less than 90°.

1.32 Streak Plate Inoculation: Use the Lid as a Shield ◆
The streak plate may be performed with the plate's base resting on the table while holding the lid over it to prevent airborne contamination. Perform the streak plate as described in the text and as shown in Figure 1.34 or Figure 1.36.



1.33 Streak Plate Inoculation: Plate Upside Down ◆
(A) Some microbiologists prefer to hold the Petri dish in the air when performing a streak plate. To do this, place the plate lid down on the table and lift the base from it, holding it on an angle. (B) Perform the streak as described in the text and as shown in Figures 1.34 and 1.36.

7 Let the loop cool for a few moments (or you can touch an open part of the agar), then perform another streak with the sterile loop beginning at one end of the first streak pattern (Fig. 1.34B). Intersect the first streak only two or three times.

8 Sterilize the loop, and then repeat with a third streak beginning in the second streak (Fig. 1.34C).

9 Sterilize the loop, and then perform a fourth streak beginning in the third streak and extending into the middle of the plate. Be careful not to enter any streaks but the third (Fig. 1.34D).

10 Sterilize the loop.

11 Label the plate's base with your name, date, and sample inoculated.

12 Incubate the plate in an inverted position for the assigned time at the appropriate temperature.

Inoculation of Agar Plates Using the T-Streak Method

The T-streak method is a variation on the quadrant streak, but only three streakings are done (Fig. 1.36). There is no particular advantage of one method over the other. It basically comes down to personal preference.

1 With a marking pen, draw one line across the plate's base about one-third of the way down the plate. Then, draw a vertical line in the larger of the two regions roughly dividing it in half. The two lines make a "T."

2 Obtain the sample of mixed culture with a sterile loop.

3 You have two options at this point. Use whichever is more comfortable for you or is required by your instructor.

a Leave the sterile agar plate on the table and lift the lid slightly, using it as a shield from airborne contamination (Fig. 1.32).

or

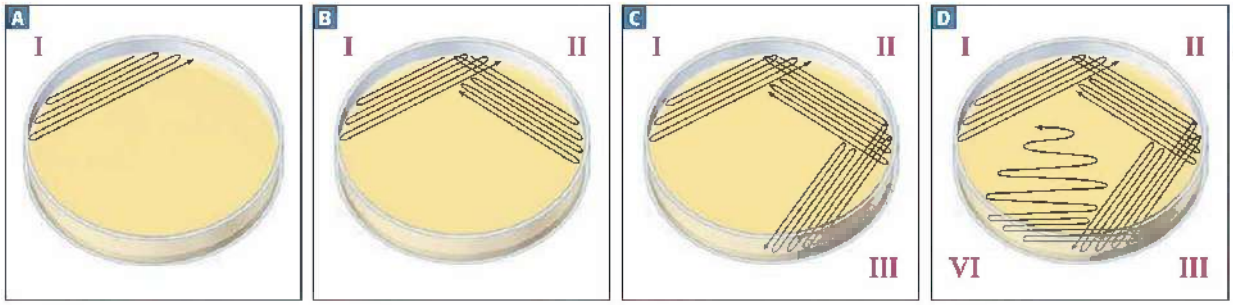
b Place the plate lid down on the table (Fig. 1.33A). Then remove the base and hold it in the air on an angle (Fig. 1.33B).

4 Streak the sample across the large region several times. Be careful not to cut the agar.

5 Flame the loop from base to tip and let it cool in the air or touch it to an uninoculated region of the agar.

6 Make two or three streaks out of the first region into the second region, and then continue with an additional four or five streaks exclusively in the second region.

7 Flame the loop from base to tip and let it cool in the air or touch it to an uninoculated region of the agar.



1.34A Beginning the Quadrant Streak Pattern ◆ Streak the mixed culture back and forth in one quadrant of the agar plate. Stay close to the plate's edge and make the streaks long. Do not cut the agar with the loop. Flame the loop, and then proceed.

1.34B Second Streak ◆ Rotate the plate nearly 90° and touch the agar in an uninoculated region to cool the loop. Streak again, using the same wrist motion. Flame the loop afterward. (***Note:*** In these illustrations, the plate is not rotated.)

1.34C Third Streak ◆ Rotate the plate nearly 90° and streak again, using the same wrist motion. Be sure to cool the loop prior to streaking and flame it afterward.

1.34D Fourth Streak Into the Center ◆ After cooling the loop, streak one last time into the center of the plate. Flame the loop, and incubate the plate in an inverted position for the assigned time at the appropriate temperature.

8. Make two or three streaks out of the second region into the third region, and then continue with an additional four or five streaks exclusively in the third region.
9. Flame the loop from base to tip.
10. Label the plate's base with your name, date, and sample inoculated.
11. Incubate the plate in an inverted position for the assigned time at the appropriate temperature.

Zigzag (Continuous) Inoculation of Agar Plates Using a Cotton Swab

This inoculation pattern is usually performed when the sample does not have a high cell density and with pure cultures when isolation is not necessary.

1. Hold the swab comfortably in your dominant hand and lift the lid of the Petri dish with the other. Use the lid as a shield to protect the agar from airborne contamination. Alternatively, the plate can be held in the air as shown in Figure 1.33B.
2. Lightly drag the cotton swab across the agar surface in a zigzag pattern, rolling it as you do so. Be careful not to cut the agar surface (Fig. 1.37).
3. Replace the lid.
4. Dispose of the swab according to your lab's practices (generally in a sharps or biohazard container).
5. Label the base of the plate with your name, date, and sample.
6. Incubate the plate in an inverted position for the assigned time at the appropriate temperature.

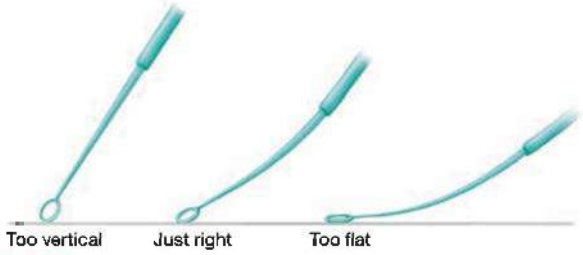
Inoculation of Agar Plates with a Cotton Swab in Preparation for a Quadrant Streak Plate

This inoculation pattern is usually performed as the initial streak for isolation of two or more bacterial species in a mixed culture with suspected high cell density.

1. Hold the swab comfortably in your dominant hand and lift the lid of the Petri dish with the other. Use the lid as a shield to protect the agar from airborne contamination (Fig. 1.33A). Alternatively, the plate can be held in the air as shown in Figure 1.33B.
2. Lightly drag the cotton swab back and forth across the agar surface in one quadrant of the plate (Fig. 1.38). This replaces the first streak as shown in Figure 1.34A.
3. Dispose of the swab according to your lab's practices (generally in a sharps container).
4. Further streaking is performed with a loop as shown in Figures 1.34B through 1.34D.
5. Label the plate's base with your name, date, and sample.
6. Incubate the plate in an inverted position for the assigned time at the appropriate temperature.

In This Exercise

You will learn how to isolate individual organisms from a mixed culture, the first step in producing a pure culture. Three related streaking techniques will be used, the choice of which is determined by the anticipated cell density of the sample.



1.35 Proper Pressure on the Loop ◆ The loop should not be held too vertically because it will cut the agar (left drawing). It also should not be held too flatly against the agar because the streaks will be too wide (right drawing). Moderate pressure should be applied to the loop so only the outer third or so of the loop's face contacts the agar (middle drawing).

✓ Materials

Per Student Group

- ☐ Broth cultures of:
  - *Micrococcus luteus*
  - *Staphylococcus epidermidis*

Per Student

- ☐ Inoculating loop
- ☐ Three tryptic soy agar (TSA) plates
- ☐ One sterile cotton swab in sterile distilled water

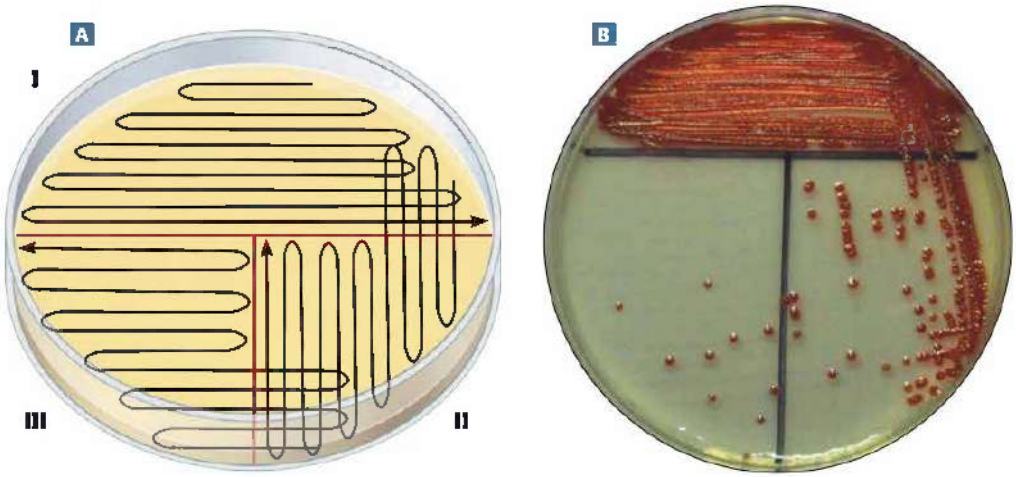
Procedure

Lab One

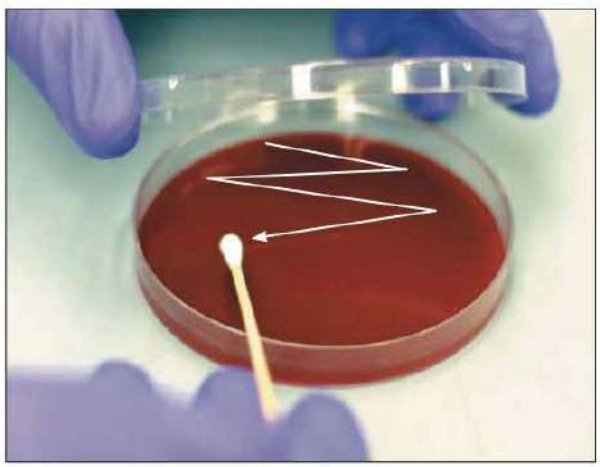
1. Using a pencil, practice quadrant-streaking the "plate" on the data sheet, page 47, before trying it with living bacteria. Rotate the paper so that each streaking motion is the same even though you are streaking a different part of the plate. ***Hint***: Keep your wrist relaxed.
2. Each student should transfer a loopful of *M. luteus* to one sterile tryptic soy agar plate and follow the diagrams in Figure 1.34 to perform a quadrant streak for isolation. Avoid digging into or cutting the agar, which ruins the plate and may create dangerous aerosols. Label the plate with your name, the date, and the organism.
3. Each student should transfer *S. epidermidis* to one sterile TSA plate and perform a T-streak for isolation (Fig. 1.36). As with the quadrant streak, avoid digging into or cutting the agar, which ruins the plate and may create dangerous aerosols. Label the plate with your name, the date, and the organism.
4. Each student should use the cotton swab to sample an environmental source (see Appendix B), and then do a simple zigzag streak on a tryptic soy agar plate (Fig. 1.37). Dispose of the swab in a sharps or biohazard container to be autoclaved. Label the plate with your name, the date, and the sample source.
5. Tape the three plates together, invert them, and incubate them at 25°C for 24 to 48 hours.

Lab Two

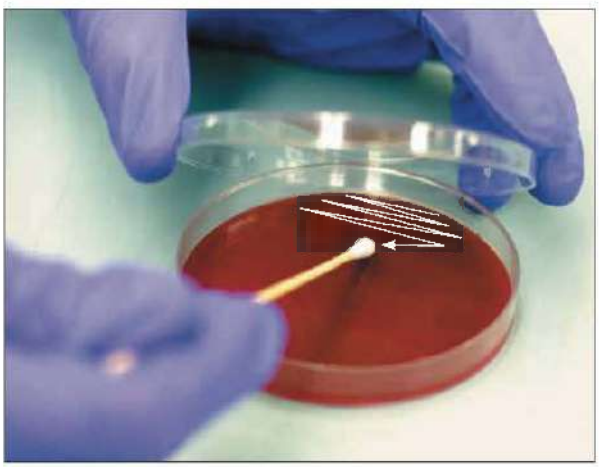
1. After incubation, examine the plates for isolation.
2. Compare your streak plates with your lab partner's plates and critique each other's technique. Remember, a successful streak plate is one that has isolated colonies; the pattern doesn't have to be textbook quality—it is just that textbook quality provides you with a greater chance of getting isolation.



1.36 T-Streak Pattern ◆ (A) This method uses only three streaks, each occupying a region designated by drawing lines in the shape of a "T" (hence, the procedure's name) on the plate's bottom (shown in red). The first region (I) is the largest (about one-third of the agar's surface) and is streaked with the original sample. After flaming the loop, a second streak is made by entering the first streak two or three times, followed by four or five streaks just in the second region (II). After flaming, a third streak is made from the second region into the third region (III) as before. (B) *Serratia marcescens* was streaked on the plate using the T-streak method. As in Figure 1.31, isolation was achieved on the second streak, which is not surprising because both plates were made from the same culture.



1.37 Zigzag (Continuous) Inoculation with a Cotton Swab ◆ Use the cotton swab to streak the agar surface to get isolated colonies after incubation. Be careful not to cut the agar. Properly dispose of the swab in a sharps or biohazard container.



1.38 Inoculation in Preparation for a Quadrant Streak ◆ If the sample is expected to have a high density of organisms, use the swab to perform the first streak toward one edge of the plate. Then continue the quadrant streak using a loop. Be careful not to cut the agar. Properly dispose of the swab in a sharps or biohazard container.

1 References

Collins, C. H. and Patricia M. Lyne. Chap. 6 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, UK: Butterworth-Heinemann, 1995.

DeMers, Marlene. Exercise 12 in *Fundamentals of Microbiology Laboratory Manual*, 8th ed. Dubuque, IA: Kendall Hunt Publishing, 2010.

Forbes, Betty A., Daniel F. Sahm, and Alice S. Weissfeld. Chap. 1 in *Bailey & Scott's Diagnostic Microbiology*, 11th ed. St. Louis, MO: Mosby-Yearbook, 2002.

Koneman, Elmer W., Stephen D. Allen, William M. Janda, Paul C. Schreckenberger, and Washington C. Winn, Jr. Chap. 2 in *Color Atlas and Textbook of Diagnostic Microbiology*, 5th ed. Philadelphia: J. B. Lippincott Company, 1997.

Power, David A. and Peggy J. McCuen. Pages 2–3 in *Manual of BBL™ Products and Laboratory Procedures*, 6th ed. Cockeysville, MD: Becton Dickinson Microbiology Systems, 1988.

Tille, Patricia M. Pages 90–91 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 1-4

Streak Plate Methods of Isolation

RESULTS AND INTERPRETATIONS

1 *Using your pencil, perform a quadrant streak on the "practice plate" below.*

2 *Examine the quadrant streak and T-streak plates. Have your lab partner write a critique of your isolation technique in the space below. The following should be addressed: Was isolation produced on one or both plates? On the quadrant streak, were the first three streaks near the edges of the plate? On both plates, did any streaks intersect streaks they shouldn't have? Was the whole surface of the agar used? Was the agar cut by the loop?*

3 *Did you achieve isolation using the quadrant streak? If so, in which streak (1, 2, 3, or 4) did it occur? If you did not achieve isolation, what might you do differently next time to improve your results?*

4 *Did you achieve isolation using the T–streak? If so, in which streak (1, 2, or 3) did it occur? If you did not achieve isolation, what might you do differently next time to improve your results?*

5 *Examine the environmental sample. Did you achieve isolation?*

6 *Were the three different streak methods appropriate to the cell densities recovered?*

7 *Most colonies on streak plates grow from isolated colony-forming units (CFUs). On rare occasions, however, a colony can be a mixture of two different organisms. If a culture is started from this colony (thinking it is pure), correct identification will be next to impossible because the extra organism could confound the identifying test results. How could you verify the purity of a colony? (The answers may vary depending on what experience you have had prior to performing this exercise.) If you found the colony to be a mixture of organisms, what could you do to purify it?*

Spread Plate Method of Isolation

Theory

The spread plate technique is a method of isolation in which a diluted microbial sample is deposited on an agar plate and spread uniformly across the surface with a glass rod. With a properly diluted sample, cells (CFUs) will be deposited far enough apart on the agar surface to grow into individual colonies.

Application

After incubation, a portion of an isolated colony can be transferred to a sterile medium to begin a pure culture. The spread plate technique also has applications in quantitative microbiology (see Section 6).

Following is a description of the spread plate technique.

Spread Plate Technique

1. Arrange the alcohol jar, Bunsen burner, and agar plate as shown in Figure 1.39. This arrangement minimizes the chances of catching the alcohol on fire.
2. Lift the plate's lid and use it as a shield to protect from airborne contamination.
3. Using an appropriate pipette, deposit the designated inoculum volume on the agar surface (Fig. 1.40). (Please see Appendices C and D for use of pipettes.) From this point, the remainder of steps should be completed within about 15 seconds to prevent the inoculum from soaking into the agar.
4. Properly dispose of the pipetting instrument used to inoculate the medium, because it is contaminated. Each lab has its own specific procedures and your instructor will advise you what to do.
5. Remove the glass spreading rod from the alcohol and pass it through the flame to ignite the alcohol (Fig. 1.41). Remove the rod from the flame and allow the alcohol to burn off completely. Do not leave the rod in the flame; the combination of the alcohol and brief flaming are sufficient to sterilize it. **Be careful not to drop any flaming alcohol on the work surface. Be especially careful not to drop flaming alcohol back into the alcohol jar. If the jar catches on fire, place the lid over it to smother the flame.**
6. After the flame has gone out on the glass rod, lift the lid of the plate and use it as a shield from airborne contamination. Then touch the rod to the agar surface away from the inoculum to cool it.

1.39 Spread Plate Set-up ◆ The spread plate technique requires a Bunsen burner, a screw-cap jar with alcohol, a glass spreading rod, and the plate. Position these components in your work area as shown: isopropyl alcohol, flame, and plate. This arrangement reduces the chance of accidentally catching the alcohol on fire. Notice the cotton in the jar's bottom to reduce the chance of breaking the glass rod.

1.40 Delivering the Inoculum ◆ Deposit the inoculum near one side of the agar surface. Use the lid as a shield and properly dispose of the pipette or pipette tip in a sharps or other biohazard container.

7 To spread the inoculum, hold the plate lid with the base of your thumb and index finger and use the tip of your thumb and middle finger to rotate the base (Fig. 1.42). At the same time, move the rod in a back-and-forth motion across the agar surface. After a couple of turns, do one last turn with the rod next to the plate's edge. Alternatively, place the plate on a rotating platform and spread the inoculum (Fig. 1.43).

1.41 Flaming the Glass Rod ◆ Remove the glass spreading rod from the alcohol jar, tap off any excess alcohol, and then pass it through the flame *away from the alcohol jar* to ignite the alcohol on it. Allow the alcohol to burn off completely. Do not leave the rod in the flame; the combination of the alcohol and brief flaming are sufficient to sterilize it. ***Be careful not to drop any flaming alcohol on the work surface or back into the alcohol jar. If the alcohol catches on fire, smother the flame by replacing the cap on the jar.***

1.42 Spreading the Inoculum ◆ After the flame has gone out on the rod, lift the lid of the plate and use it as a shield from airborne contamination. Then, touch the rod to the agar surface away from the inoculum in order to cool it. To spread the inoculum, hold the plate lid with the base of your thumb and index finger, and use the tip of your thumb and middle finger to rotate the base. At the same time, move the rod in a back-and-forth motion across the agar surface. After a couple of turns, do one last turn with the rod next to the plate's edge.

8 Remove the rod from the plate and replace the lid.

9 Return the rod to the alcohol in preparation for the next inoculation. There is no need to flame it again.

10 Label the plate base with your name, date, organism, and any other relevant information.

11 Incubate the plate in an inverted position at the appropriate temperature for the assigned time. (If you plated a volume of inoculum greater than 0.5, wait a few minutes and allow it to soak in before inverting the plate.)

In This Exercise

You will perform a spread plate inoculation. In the context of this exercise, it is used as an isolation procedure, but it also can be used in quantifying cell densities in broth samples.

✓ Materials

Per Student Pair

- ☐ Inoculating loop (each student)
- ☐ Six sterile plastic transfer pipettes
- ☐ Glass spreading rod (alternatively, sterile disposable plastic spreaders are available)
- ☐ Glass, screw-cap jar with cotton in the bottom, and enough isopropyl alcohol to only cover the spreading part of the glass rod (not necessary if disposable spreaders are used)

1.43 Spreading with a Turntable ◆ An inoculating turntable makes it easier to rotate the plate during the spread plate technique.

- ☐ Bunsen burner and striker
- ☐ Four nutrient agar plates
- ☐ One sterile microtube
- ☐ Four capped microtubes with about 1 mL sterile distilled or deionized water (dH_2O)
- ☐ (Optional) vortex mixer
- ☐ Broth cultures of:
  - *Escherichia coli*
  - *Serratia marcescens*

Procedure

Lab One

1. Using a different pipette for each, transfer a few drops of *E. coli* and *S. marcescens* to a microtube. Cap the tube and mix well with a vortex mixer. Or use the second pipette to mix well by gently drawing and dispensing the mixture in and out of the tube a couple of times. Do not spray the mixture!
2. Label the four microtubes containing sterile dH_2O "A," "B," "C," and "D."
3. Label the four nutrient agar plates "A," "B," "C," and "D."
4. Transfer a loopful of the mixture from the microtube to Tube A and mix well with the loop or a vortex mixer. If using a vortex mixer, be sure to cap the tube.[1]
5. Transfer a loopful of the mixture in Tube A to Tube B and mix well with the loop or a vortex mixer.
6. Transfer a loopful of the mixture in Tube B to Tube C and mix well with the loop or a vortex mixer.
7. Transfer a loopful of the mixture in Tube C to Tube D and mix well with the loop or a vortex mixer.
8. Using a sterile transfer pipette, place a couple of drops of sample from Tube A on Plate A. Spread the inoculum with a glass rod as described in "Spread Plate Technique" and as shown in Figures 1.40 through 1.42. Let the plate sit for a few minutes.
9. Repeat step 8 for Tubes B, C, and D and Plates B, C, and D, respectively.
10. Tape the four plates together (be sure they are facing the same direction), invert them and incubate them at 25°C for 24 to 48 hours.

Lab Two

1. After incubation, examine the plates for isolation. *S. marcescens* produces reddish-orange colonies, and *E. coli* produces buff-colored colonies.
2. Complete the data sheet on page 53.

[1] Because this is not a quantitative procedure, it is not necessary to flame the loop between transfers.

References

Clesceri, WEF, Chair; Arnold E. Greenberg, APHA; Andrew D. Eaton, AWWA; and Mary Ann H. Franson. Pages 9–38 in *Standard Methods for the Examination of Water and Wastewater*, 20th ed. Washington, DC: Joint publication of American Public Health Association, American Water Works Association, and Water Environment Federation. APHA Publication Office, 1998.

Downes, Frances Pouch, and Keith Ito. Page 57 in *Compendium of Methods for the Microbiological Examination of Foods*. Washington, DC: American Public Health Association, 2001.

Gerhard, Philipp, R. G. E. Murray, Willis A. Wood, and Noel R. Kreig. Pages 255–257 in *Methods for General and Molecular Bacteriology*. Washington DC: American Society for Microbiology, 1994.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 1-5

Spread Plate Method of Isolation

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations in the table below.

Organism	Plate(s) with Isolation	Comments
E. coli		
S. marcescens		

QUESTIONS

1 *Think about your results in attempting to isolate* E. coli *and* S. marcescens.

a. *On which plate did you first obtain isolation with* E. coli*?*

b. *On which plate did you first obtain isolation with* S. marcescens*?*

c. *Do you have reason to suspect that they should become isolated on the same dilution plate? Why or why not?*

2 *Once you obtained isolation at a particular dilution, did you continue to have isolation on subsequent dilution plates? Is this what you would expect? Why or why not?*

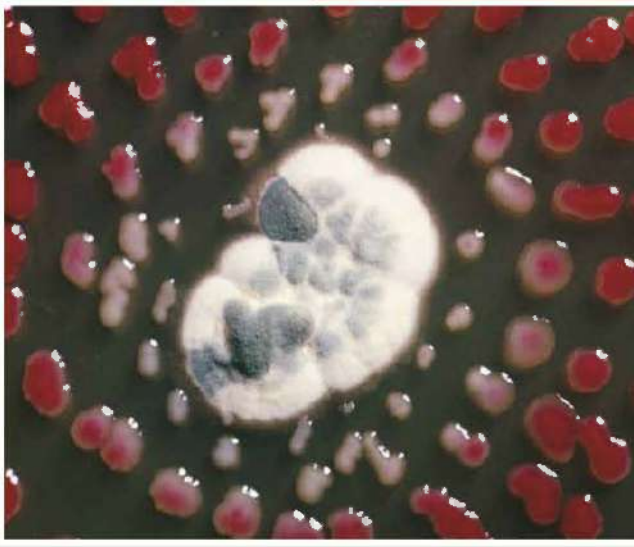
3 *What is the primary* negative *consequence of not spreading the inoculum evenly over the agar surface?*

4 *To get isolated colonies on a plate, only about 300 cells can be in the inoculum. What will happen if the cell density of the inoculum significantly exceeds this number?*

5 *Suppose you have two organisms in a mixture and Organism A is 1,000 times more abundant than Organism B. Will you (without counting on good luck!) be able to isolate Organism B using the spread plate technique? Explain your answer.*

SECTION 2

Microbial Growth



Microorganisms are extraordinarily diverse, and every species demonstrates a unique combination of characteristics, some of which can be easily observed. In this section we illustrate some of those characteristics and factors that affect them.

You will begin this section with an exercise intended to sensitize you to the diversity of microbial populations living all around us. Allowing for variables, such as the growth medium and incubation conditions, much can be determined about an organism by simply looking at the colonies it produces, or its appearance on slants or in broths. Distinguishing growth patterns on or in different media is an important skill—one that you can use as you progress through the semester. Note the growth characteristics of all the organisms provided for your laboratory exercises, and jot them down or even sketch them. When the time comes to identify your unknown species, you may find your records very useful.

Next, you will examine microbial nutritional diversity by growing bacteria on media with varying amounts of carbon and nitrogen resources. Following that, you will look at some environmental factors affecting microbial growth, such as oxygen, temperature, pH, and osmotic pressure. Finally, you will examine some physical and chemical microbial control agents and systems, that is, ways in which humans can control bacterial growth.

Diversity and Ubiquity of Microorganisms

Microorganisms are found everywhere that other forms of life exist. In Exercise 2-1, you will transfer microorganisms from seemingly uninhabited sources and grow them on agar plates. Each source is likely to have multiple species growing on or in it and represents a **mixed culture**. When you inoculate the plates you will be transferring untold numbers of unknown cells to them. Any cells that are able to grow on the plate's medium will divide and produce visible **colonies** of identical cells.

In Exercise 2-2 you will learn to identify some of the various growth characteristics produced by the "invisible" cohabitants from Exercise 2-1, as well as examining plates of known species. An ability to recognize differences **in colony morphology** is often the first clue to a microbiologist that two organisms are different species. If a colony is not contacting other colonies, it is said to be **isolated** and a portion of it can be transferred to a sterile medium to start a **pure culture** of the species, which is then frequently used in identifying the species. Pure cultures are usually grown in a broth or on a slanted medium. In Exercises 2-3 and 2-4 you will examine growth characteristics of bacteria on slants and in broth, respectively. ◆

EXERCISE 2-1

Ubiquity of Microorganisms

Theory

Microorganisms have a long, rich history on Earth and have successfully adapted to a wide range of habitats. The literature on microorganisms often describes them as being "ubiquitous in nature." More specifically, this means that microorganisms of all sorts can be isolated from soil (of all sorts), water (over a large range of salinities), plants, and animals (including humans). Microbes are even found in apparently uninhabitable sites such as hot acid pools (Sulfur Caudron in Yellowstone is approximately 85+°C with a pH close to 1!).

Many microorganisms are **free-living**—they do not reside on or in a specific plant or animal **host** and are not known to cause disease. They are **nonpathogenic.** Frequently they are **saprophytes** and perform the important ecosystem role of decomposing organic matter. Other microorganisms reside on or in another species and benefit from the symbiotic association with their host(s)—they have a place to live and reproduce successfully. If they cause damage to their host, that is, they cause disease, they are **pathogens** of it. In other instances the microbe may actually benefit their host, an example of **mutualism.** Lastly, some microbes are **commensals,** where they benefit but have no significant effect on their host. However, even many of the commensal or mutualistic strains inhabiting our bodies are **opportunistic pathogens.** That is, they are capable of producing a disease state if introduced into a suitable part of the body. Any area, including sites outside of the host organism, where a microbe resides and serves as a potential source of infection is called a **reservoir.**

Application

This exercise is designed to demonstrate the ubiquitous nature of microorganisms and the ease with which they can be cultivated. (It should be noted that although we can find living microorganisms virtually everywhere and confirm their presence by cultivation, molecular techniques developed over the last two or three decades demonstrate that the organisms successfully grown in the lab represent a minute fraction of those still uncultivable.)

In This Exercise

Today, you will work in small groups to sample and culture several locations in your laboratory. Your instructor may have other locations outside of the lab to sample as well. Remember that even relatively "harmless" bacteria, when cultivated on a growth medium, are in sufficient enough numbers to constitute a health hazard. Treat them with care.

✓ Materials

Per Student Group

- ☐ Eight nutrient agar plates
- ☐ One sterile cotton swab

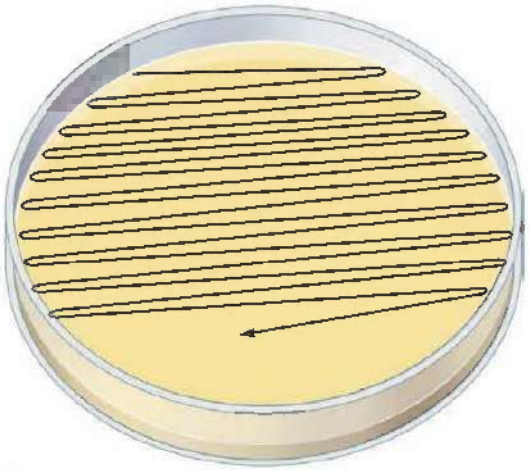
2

Procedure

Lab One

1. Number the plates 1 through 8.
2. Open plate number 1 and expose it to the air for 30 minutes or longer. Set it aside and out of the way of the other plates.
3. Use the cotton swab to sample your desk area and then streak plates 2 and 3 in the pattern shown in Figure 2.1. Press very lightly as you roll the swab on the table top and then again on the agar to inoculate it. Be sure the contaminated part of the swab contacts the agar so you actually transfer any organisms collected.
4. Hold the plate directly in front of your mouth and cough several times on the agar surface of plate 4. Be sure you aren't facing anyone when you cough. In fact, it is best to cough downward onto the plate to minimize spreading germs throughout the classroom. Send them to the floor instead!
5. Rub your hands together, and then touch the agar surface of plate 5 lightly with your fingertips. A light touch is sufficient; touching too firmly will crack the agar. Your fingers will feel gooey afterward, but the agar is sterile so there should actually be *fewer* microbes on your fingers than before you touched it.
6. Remove the lid of plate 6 and vigorously scratch your head above it. It's best to bend your head forward so your hair is actually above the plate. When inoculating, do so away from plate 1 to avoid cross-contamination.
7. Leave plates 7 and 8 covered; do not open them.
8. Label the base of each plate with the date, type of exposure it has received, and the name or number of your group.
9. Invert all plates and incubate them for 24 to 48 hours at the following temperatures:

Plates 1, 2, and 8:	25°C
Plates 3, 4, 5, 6, and 7:	37°C



2.1 Simple Streak Pattern on an Agar Plate ◆ Roll the swab as you collect the sample from the table and then again as you inoculate the plate. Do not press so hard that you cut the agar.

Lab Two

1. Using the plate diagrams on the data sheet, page 59, draw two representative colonies on each of your agar plates. Be sure to label them according to incubation time, temperature, and source of inoculum. It is best to select one colony from each plate to draw before doing the second one. That way you will have observed each plate in case time runs short. *Note:* Do not remove the plate's lid if there is fuzzy growth on it or your instructor has told you not to. If you are in doubt about "fuzzy growth," ask your instructor.
2. Save these plates in a refrigerator for use in Exercise 2-2.

References

Holt, John G., ed. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Baltimore: Lippincott Williams & Wilkins, 1994.

"Sulphur Caldron." Available online. URL: http://www.yellowstoneparknet.com/geothermal_features/sulphur_caldron.php.

Tille, Patricia M. Chap. 7 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Varnam, Alan H. and Malcolm G. Evans. *Environmental Microbiology*. Washington, DC: ASM Press, 2000.

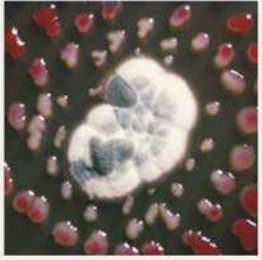
Winn, Washington C. et al. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-1

Ubiquity of Microorganisms

OBSERVATIONS AND INTERPRETATIONS

1 Use the circles below as Petri dishes. Then, for each plate, choose two different colonies and draw each as seen from above and from the side. Simple line drawings are acceptable, but do them with care. Chicken scratches are not very useful! Label the plates according to incubation time, temperature, and source of inoculum. Also include other useful colony information, such as color and relative abundance.

2 Save the plates for Exercise 2-2.

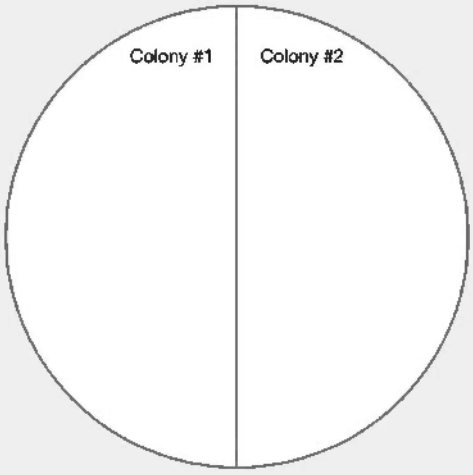


Plate #1 _____

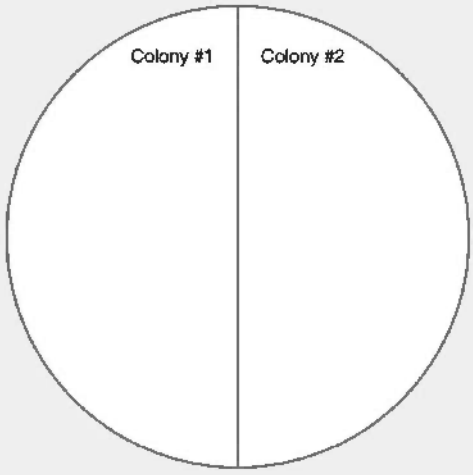


Plate #2 _____

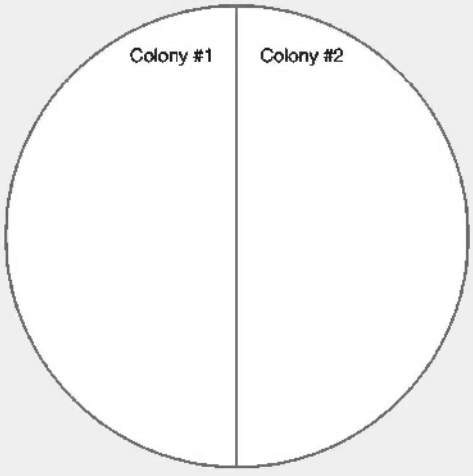


Plate #3 _____

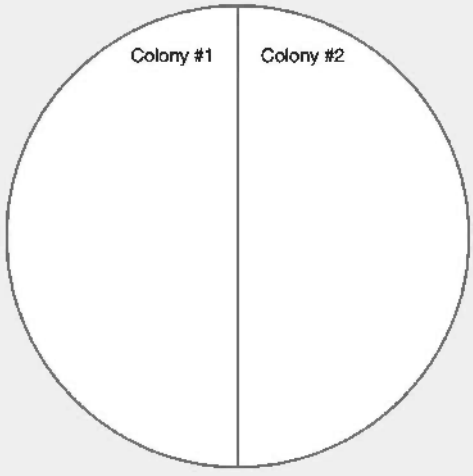


Plate #4 _____

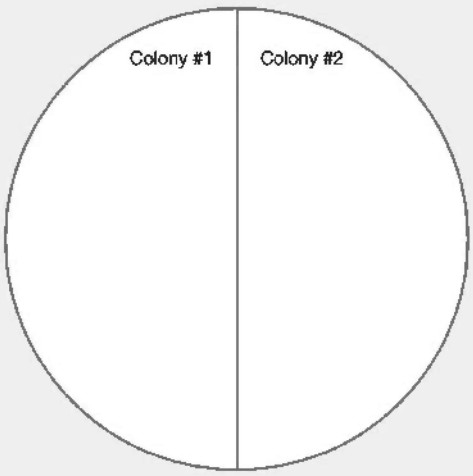


Plate #5 _____

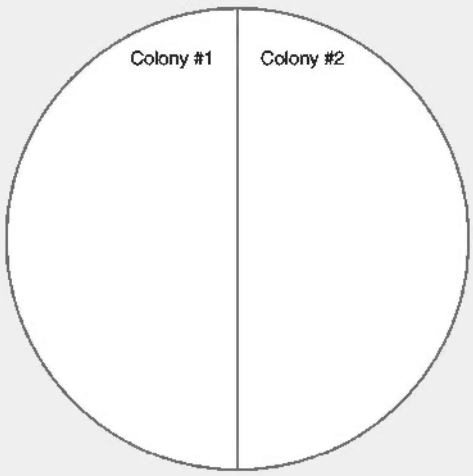


Plate #6 _____

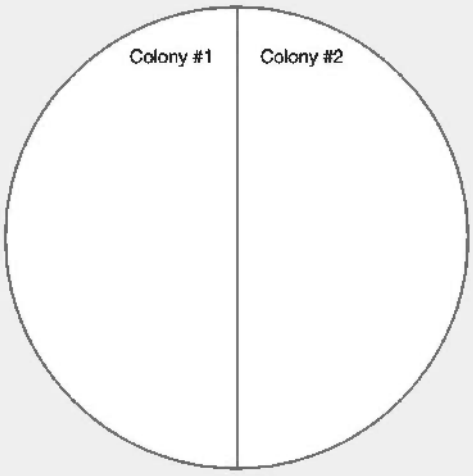


Plate #7 _____

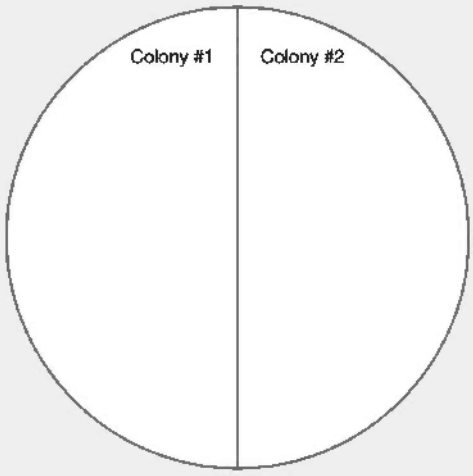


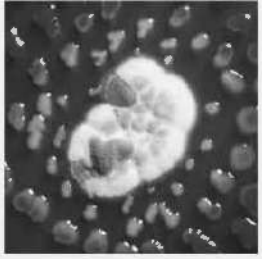
Plate #8 _____

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-1 *(continued)*

QUESTIONS

1 *Consider plates 7 and 8.*

a. *What was the purpose of incubating the unopened plates? Be specific.*

b. *What is an appropriate name for these plates?*

c. *If growth appears on both unopened plates, what are some likely explanations?*

d. *What if growth appears on only one plate?*

e. *How does growth on the unopened plates affect the reliability (your interpretation) of the other plates?*

2 *Why do you think the specific types of exposure (air, hair, tabletop, etc.) were chosen for this exercise?*

3 *Consider plates 2 and 3.*

a. *Did you get different-appearing colonies on plates 2 and 3? If so, explain why.*

b. *What is the likely source (reservoir) of organisms that grew best at 37°C, and how do they survive at room temperature without nutrients?*

4 *Suppose plate 4 (cough) has no growth after incubation. It is highly unlikely the "cougher" has sterile coughs! Suggest reasons why no growth was recovered on the plate.*

5 *The plates you are using for this lab will be autoclaved to completely sterilize them. The measures taken to disinfect the tabletops (the source of the organisms on plates 2 and 3) are not as extreme. Why?*

Colony Morphology

EXERCISE 2-2

Theory

When a single bacterial cell is deposited on a solid nutrient medium, it begins to divide. One cell makes two, two make four, four make eight . . . one million make two million, and so on. Eventually a visible mass of cells—a **colony**—appears where the original cell was deposited. Color, size, shape, and texture of microbial growth are determined by the genetic makeup of the organism (in many cases by yet unknown mechanisms), but are also greatly influenced by environmental factors including nutrient availability, temperature, and incubation time.

Colony morphological characteristics may be viewed with the naked eye, a hand lens, or a colony counter (Fig. 2.2). The basic categories include colony shape, margin (edge), surface, elevation, texture, and optical properties (Fig. 2.3).

1. *Shape* may be described as **round**, **irregular**, or **punctiform** (tiny, pinpoint).
2. The *margin* may be **entire** (**smooth**, with no irregularities), **undulate** (wavy), **lobate** (lobed), **filamentous** (unbranched strands), or **rhizoid** (branched like roots).
3. The *surface* may be **smooth**, **rough**, or **wrinkled**.
4. *The texture* may be **moist**, **mucoid** (sticky), **butyrous** (buttery), **dry**, **shiny**, or **dull**.
5. *Elevations* include **flat**, **raised**, **convex**, **pulvinate** (very convex), and **umbonate** (raised in the center).
6. Other useful features include **color** and optical properties such as **opaque** (you can't see through it) and **translucent** (light passes through).

Features such as colony shape, margin, surface, and texture (shiny or dull) are best viewed while holding the plate level with the lid off, but rocking it back and forth slightly so reflected light hits it at different angles. Elevations are best viewed with the plate tilted slightly at eye level. Opacity and translucence are best viewed by holding the plate (lid on) so it is illuminated from behind (or by placing it on a colony counter). If allowed to do so, you may check texture by touching the growth with an inoculating loop or wooden stick. Be sure to flame the loop afterward or dispose of the wooden stick properly.

Application

Recognizing different bacterial growth morphologies on agar plates is a useful step in the identification process. It is often the first indication that one organism is different from another. Once purity of a colony has been confirmed by an appropriate staining procedure (this is not always done), cells can be transferred to a sterile medium, grown, and maintained as a pure culture, which then acts as a source of that microbe for identification or other purposes.

In This Exercise

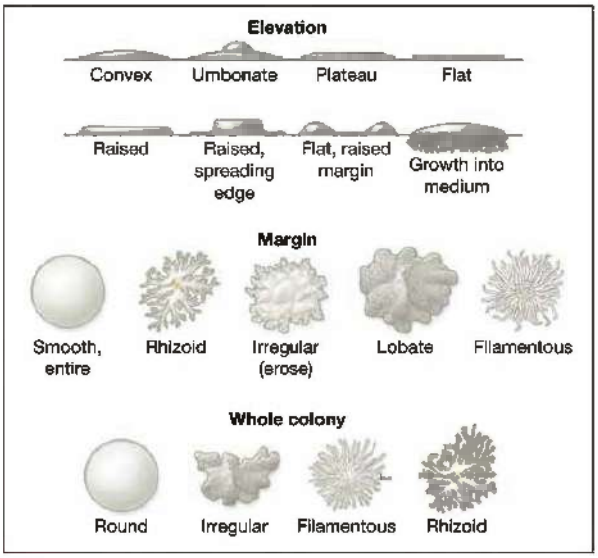
Today you will be viewing colony characteristics on the plates saved from Exercise 2-1 and (if available) prepared streak plates provided by your instructor. Figures 2.4 through 2.33 show a variety of bacterial colony forms and characteristics. Where applicable, contrasting environmental factors are indicated.

2.2 Colony Counter ◆ Subtle differences in colony shape and size can best be viewed on the colony counter. The **transmitted light** and magnifying glass allow observation of greater detail; however, colony color and many other features are best determined with **reflected light**. The grid in the background is a counting aid; each big square is 1 square centimeter.

✓ Materials

Per Student Group

- □ (Optional) colony counter, dissecting microscope, or hand lens
- □ Metric ruler
- □ Plates from Exercise 2-1
- □ (Optional) Tryptic soy agar or brain-heart infusion agar streak plate cultures of any of the following:
  - *Bacillus subtilis*
  - *Corynebacterium xerosis*
  - *Kocuria rosea*
  - *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - *Micrococcus luteus*
  - *Mycobacterium smegmatis* (BSL-2)
  - *Proteus mirabilis* (BSL-2)

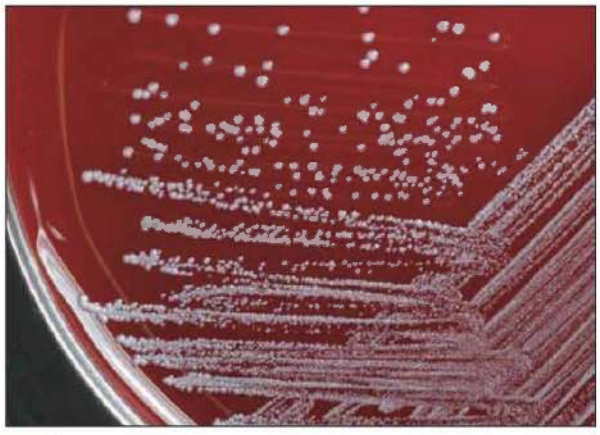


2.3 A Sampling of Bacterial Colony Features ♦ These terms are used to describe colonial morphology. Descriptions also should include color, size, surface characteristics, texture, and optical properties (opaque or translucent). See the text for details.

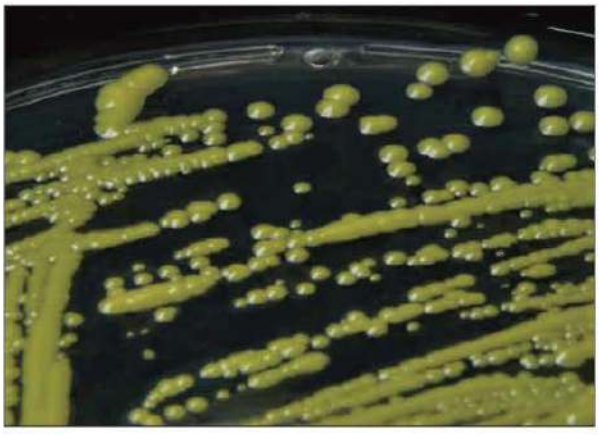
Procedure

1. Using the terms in Figure 2.3 and in the text, describe some representative colonies on your plates from Exercise 2-1 (if not already described) and the pure cultures supplied. Measure colony diameters (in mm) with a ruler and include them with your descriptions in the table on the data sheet, page 73. If you see a distinctive feature that has not been given a name, make up one! Just make it descriptive and easily understood by others. That's what the early microbiologists did to compile the list you have been given. (*Note:* Remember that many microorganisms are opportunistic pathogens, so be sure to handle the plates carefully. **Use BSL-2 precautions when transferring *Mycobacterium smegmatis* and the *Proteus mirabilis*, if used.** Do not open plates with BSL-2 organisms on them or those containing fuzzy growth, because a fuzzy appearance suggests fungal growth containing spores that can spread easily and contaminate the laboratory and other cultures. If you are in doubt, check with your instructor.)
2. Unless you have been instructed to save today's cultures for future exercises (such as Exercise 2-3), discard all plates in an appropriate autoclave container.

2.4 *Enterococcus faecium* Grown on Nutrient Agar ♦ The colonies are white, circular, convex, and have an entire margin. *E. faecium* (formerly known as *Streptococcus faecium*) is found in human and animal feces.



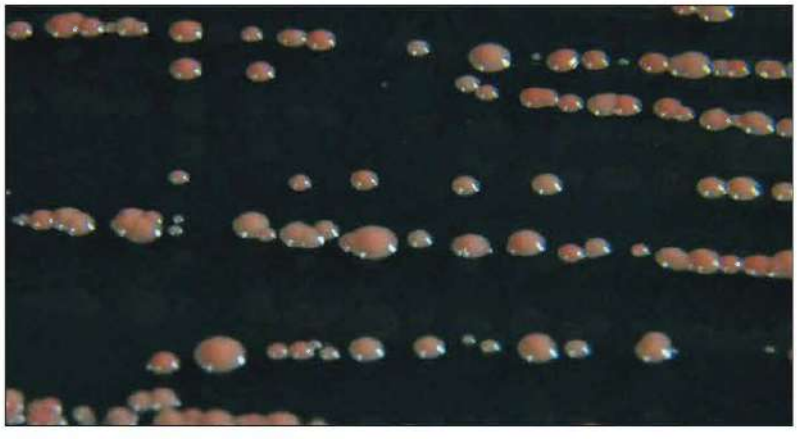
2.5 ***Staphylococcus epidermidis*** **Grown on Sheep Blood Agar** ♦ The colonies are white, raised, circular, and entire. *S. epidermidis* lives on human skin (as the name indicates) and is an opportunistic pathogen.



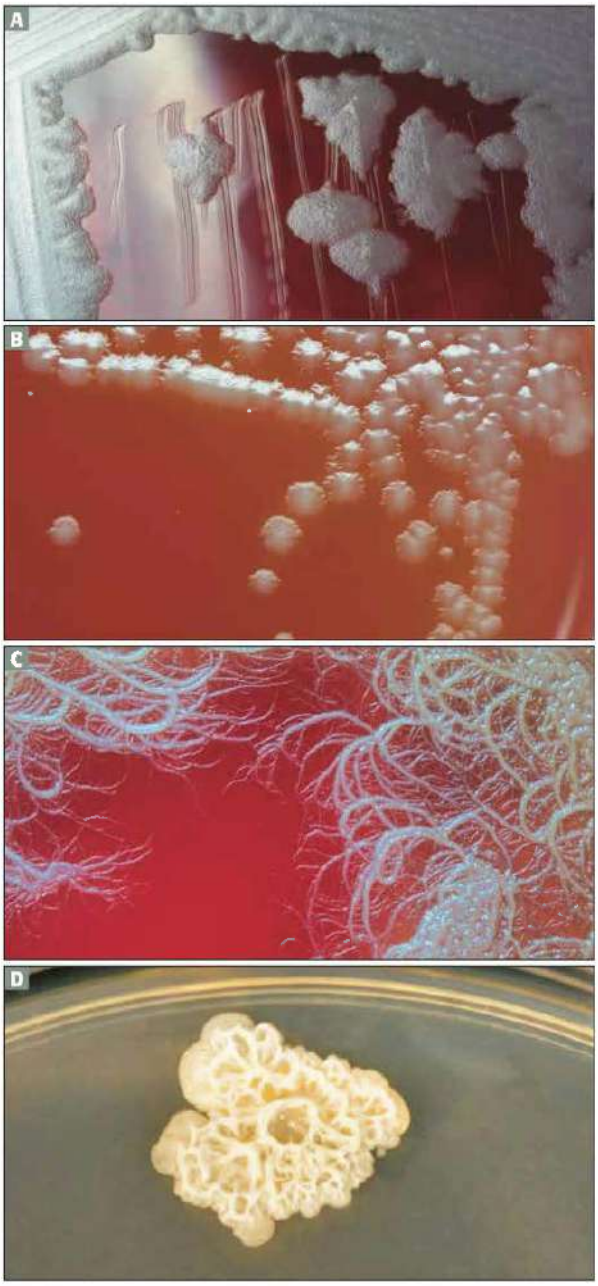
2.6 ***Micrococcus luteus*** **grown on Brain-Heart Infusion Agar** ♦ These colonies are yellow, smooth, and convex with an entire margin. They range in size from 1 mm to 3 mm. *M. luteus* (*luteus* means "yellow") is common in soil, dust, and on human skin.

2.7 ***Kocuria rosea*** **Grown on Brain-Heart Infusion Agar** ♦ Pink (hence "*rosea*"), punctiform (these are less than 1 mm in diameter), smooth, regular colonies typify *K. rosea*, and are inhabitants of water, dust, and salty foods.

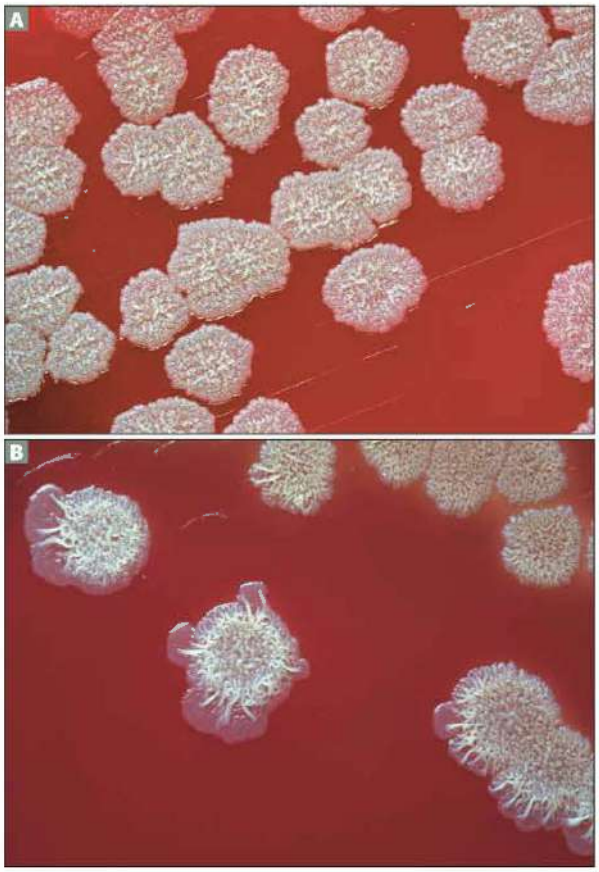
2.8 ***Sarcina aurantiaca*** **Grown on Brain-Heart Infusion Agar** ♦ *S. aurantiaca* (*aurantiaca* refers to "golden") produced yellow-orange, smooth, entire, convex colonies on BHIA. These are between 1 mm and 3 mm in diameter.



2.9 ***Rhodococcus rhodochrous*** **Grown on Brain-Heart Infusion Agar** ♦ These colonies are reddish-pink (hence "rhodo"), smooth, and convex with an entire margin. They are about 1 mm in diameter. *Rhodococcus* species are soil organisms.



2.10 Comparison of Four *Bacillus* Species ♦ **(A)** *B. cereus* grown on sheep blood agar (SBA) produces distinctively large (up to 7 mm), gray, granular, irregular colonies. They often produce a "mousy" smell. Also note the distinctive extensions of growth along the streak line. **(B)** *B. anthracis* colonies on SBA resemble *B. cereus*, but are usually smaller and adhere to the medium more tenaciously. It must be handled at *least* in a BSL-2 laboratory, and sometimes BSL-3 if the cell density is high enough, so it is unlikely you will be testing its tenacity! **(C)** *B. mycoides* produces distinctive, rapidly spreading, rhizoid (note the branching) colonies. It is a common isolate from soil and is shown here on SBA. **(D)** This unknown *Bacillus* isolated as a laboratory contaminant produced a wrinkled, irregular colony with an irregular (wavy) margin on tryptic soy agar.



2.11 Effect of Age on Colony Morphology ♦ **(A)** Close-up of *Bacillus subtilis* on sheep blood agar after 24 hours of incubation. **(B)** Close-up of *Bacillus subtilis* on sheep blood agar after 48 hours of growth. Note the wormlike appearance.

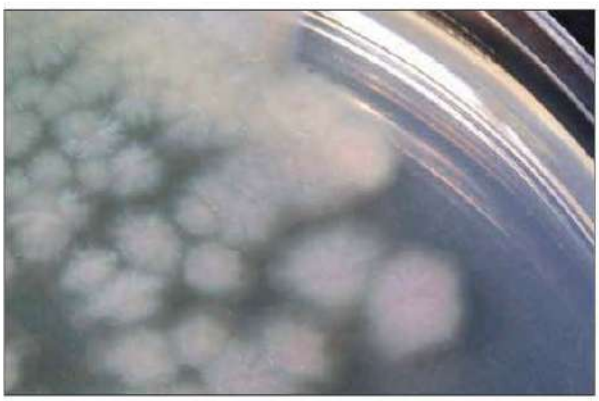
2.12 *Clostridium sporogenes* Grown Anaerobically on Sheep Blood Agar ♦ These irregularly circular colonies have a raised center and a flat, spreading edge of tangled filaments (reminiscent of the mythological creature Medusa, who had snakes for hair!). They vary in size from 2 mm to 6 mm. *C. sporogenes* is found in soils worldwide.

2.13 ***Providencia stuartii*** **Grown on Nutrient Agar** ♦ The colonies are shiny, buff, and convex. *P. stuartii* is a frequent isolate in urine samples obtained from hospitalized and catheterized patients. It is highly resistant to antibiotics.

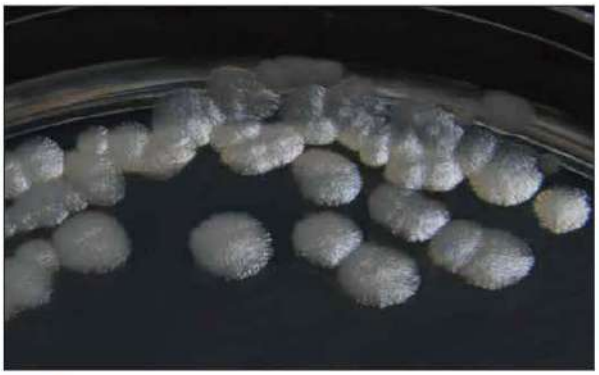
2.14 ***Klebsiella pneumoniae*** **Grown on Nutrient Agar** ♦ The colonies are mucoid, raised, and shiny. While it is a normal inhabitant of the human intestinal tract, it is associated with community-acquired pneumonia and nosocomial urinary tract infections.

2.15 ***Alcaligenes faecalis*** **Grown on Sheep Blood Agar** ♦ The colonies of this opportunistic pathogen are umbonate with an opaque center and a spreading edge. (**A**) Side view: note the raised center. (**B**) Close-up of *A. faecalis* colonies showing spreading edge.

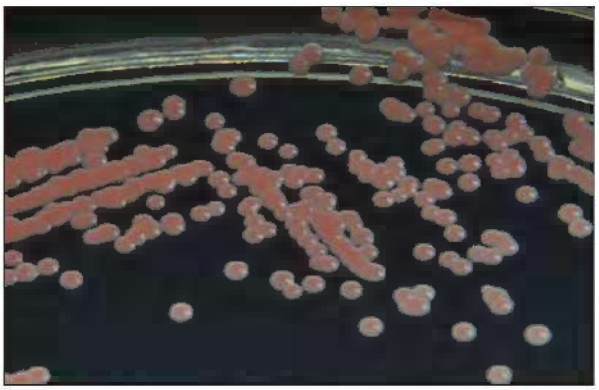
2.16 ***Citrobacter koseri*** **Grown on Sheep Blood Agar** ♦ The colonies are round, smooth, and opaque with an entire margin. This species is also able to partially hemolyze (break down) red blood cells (it is α-hemolytic), as evidenced by the greening around each colony. They range in size from 1 mm to 2 mm.



2.17 ***Erwinia amylovora*** **Grown on Nutrient Agar** ♦ Note the irregular shape and spreading edges. *E. amylovora* is a plant pathogen. Its scientific name literally means "Erwin (Smith's) starch devourer."

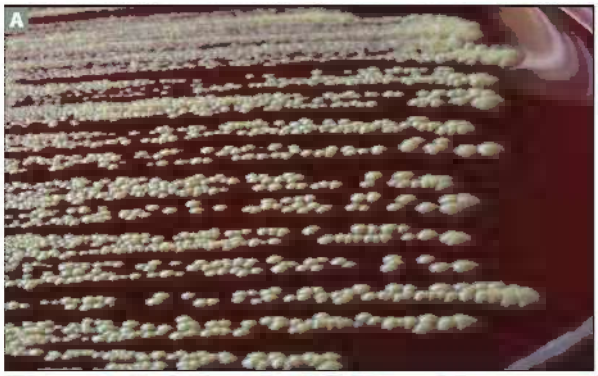


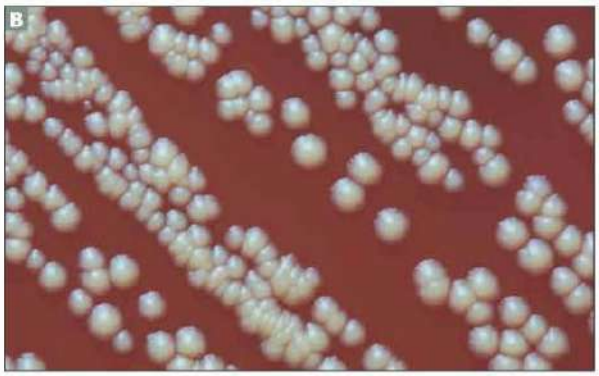
2.18 ***Rhizobium leguminosarum*** **Grown on Brain-Heart Infusion Agar** ♦ The colonies are convex, circular, and filamentous. They are translucent at the edges and about 5 mm in diameter. *R. leguminosarum* is capable of causing root nodule formation (*rhiz–* means "root") in many legumes and subsequently fixing atmospheric nitrogen.



2.19 ***Deinococcus radiodurans*** **Grown on Tryptic Soy Agar** ♦ These small (between 1 mm and 2 mm in diameter), round, convex, and entire colonies took 36 hours to develop their orange color. This species is highly resistant to ionizing radiation.

2.20 ***Mycobacterium smegmatis*** **Grown on Sheep Blood Agar** ♦ The colonies of this slow-growing relative of *M. tuberculosus* are punctiform.



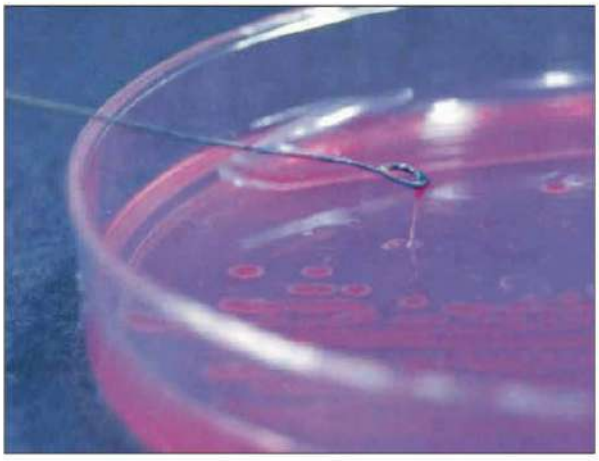


2.21 ***Corynebacterium xerosis*** **Grown on Sheep Blood Agar** ♦ (A) As seen in this view from the side, the colonies are round, dull, buff, and convex. (B) Close-up of circular *C. xerosis* colonies. *C. xerosis* is rarely an opportunistic pathogen.

2.22 **Umbonate Colonies of an Anaerobic Lab Contaminant Grown on Tryptic Soy Agar** ♦ The colony on the left is truly umbonate. The one on the right is getting there. Their diameters are about 3 mm.

2.23 ***Streptomyces griseus*** **Grown on Brain-Heart Infusion Agar** ♦ These colonies are circular, entire, and granular with a ridged surface. At a later stage of development, they produce yellow reproductive spores. Growth of streptomycetes is associated with an "earthy" smell. This one plate fragranced the entire incubator!

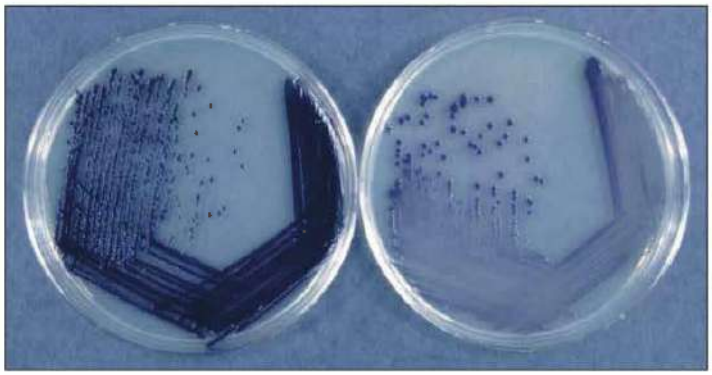
2.24 Swarming Growth Pattern ♦ Members of the genus *Proteus* will swarm at certain intervals and produce a pattern of concentric rings because of their motility. This photograph demonstrates the swarming behavior of *P. vulgaris* on DNase agar.



2.25 Mucoid Colonies of *Pseudomonas aeruginosa* ♦ *Pseudomonas aeruginosa* grown on Endo agar illustrates a mucoid texture. *P. aeruginosa* is found in soil and water and can cause infections in burn patients.

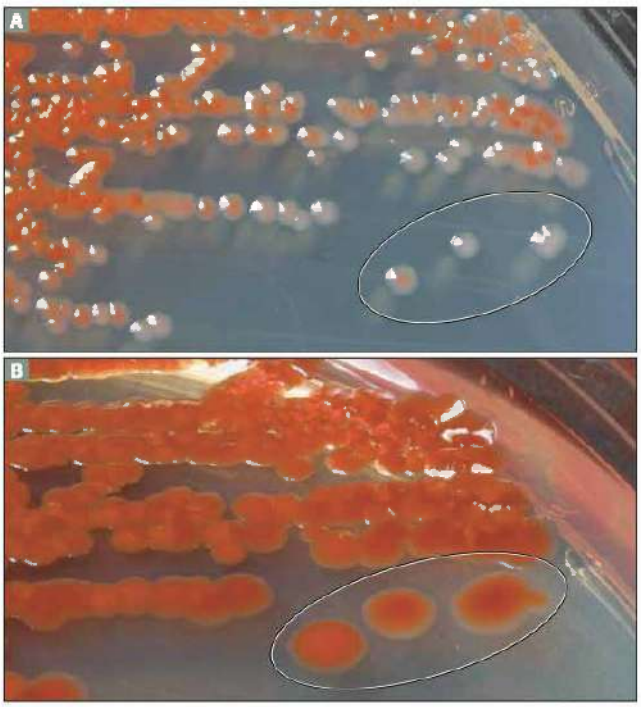
2.26 Butyrous Colony of an Unknown Soil Isolate ♦ This 12 mm colony was found on a glycerol yeast extract plate inoculated with a diluted soil sample. It was almost liquid in texture, something that is demonstrated by its contact with the yellow colony to its right.

2.27 *Chromobacterium violaceum* Grown on Sheep Blood Agar ♦ *C. violaceum* produces shiny, purple (hence "violaceum"), convex colonies. It is found in soil and water, and rarely produces infections in humans.



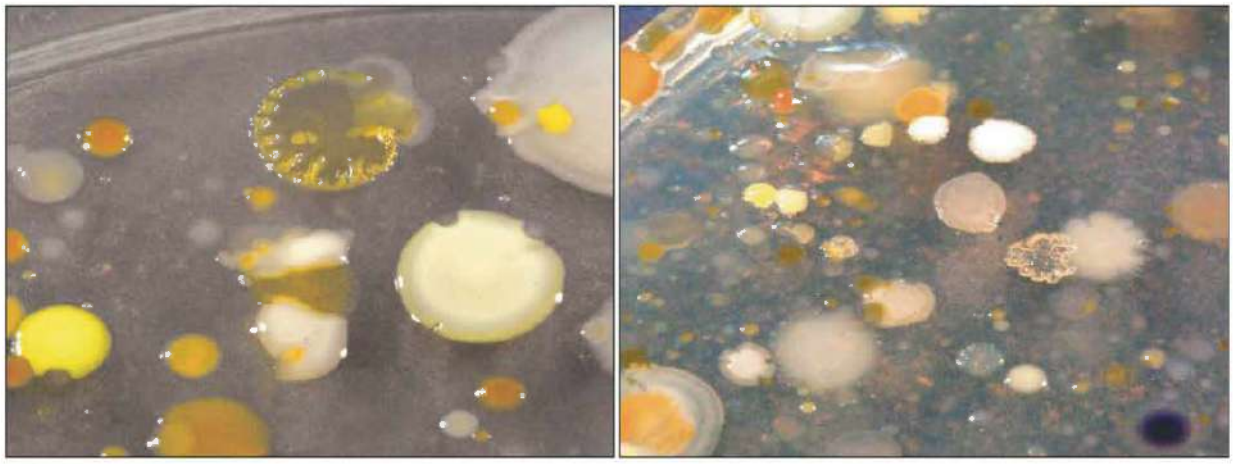
2.28 Influence of Nutrient Availability on Pigment Production ♦ Pigment production may be influenced by environmental factors such as nutrient availability. *Chromobacterium violaceum* produces a much more intense purple pigment when grown on tryptic soy agar (left) than when grown on nutrient agar (right), a less nutritious medium.

2.29 Influence of Incubation Temperature on Pigment Production ♦ Pigment production may be influenced by temperature. *Serratia marcescens* produces less orange pigment when grown at 37°C (left) than when grown at 25°C (right).

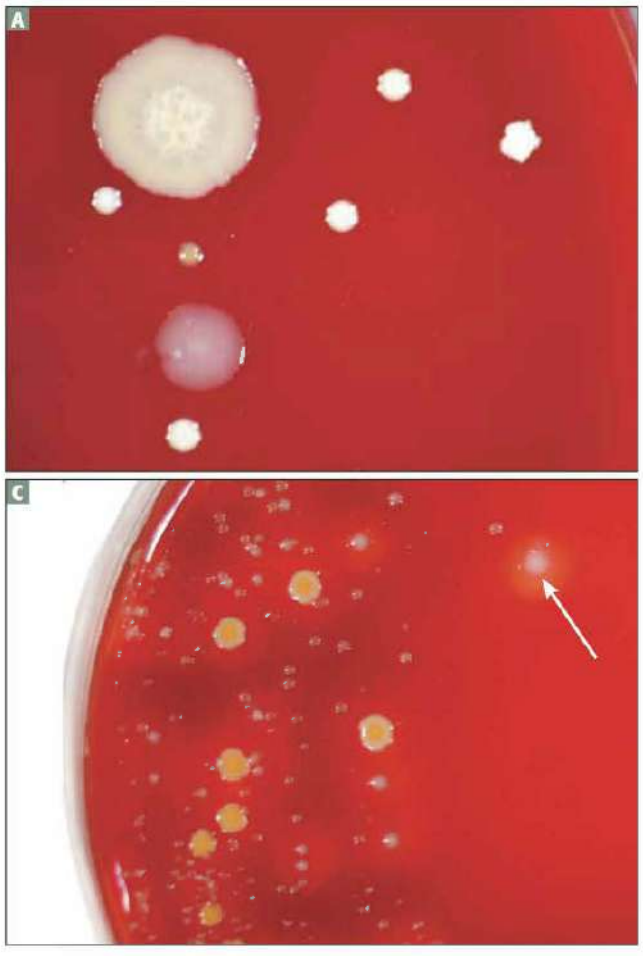


2.30 Influence of Culture Age on Pigment Production ♦
(A) *Serratia marcescens* grown on nutrient agar after 24 hours. (B) The same plate of *S. marcescens* after 48 hours. Note in particular the change in the three colonies in the lower right (encircled).

2.31 Diffusible Pigment of *Pseudomonas aeruginosa* ♦
The blue-green pigment pyocyanin diffusing from the growth is distinctive of *P. aeruginosa*. Here, *P. aeruginosa* is growing on tryptic soy agar.



2.32 Two Mixed Soil Cultures on Nutrient Agar ♦ These plates show the morphological diversity present in two diluted soil samples.



2.33 Three Throat Cultures Grown on Sheep Blood Agar ♦ (**A**) There are probably five different species on this plate. (**B**) Note the α-hemolysis (darkening of the agar) shown by much of the growth. (**C**) This is a close-up of the same plate as in **B**. Note the weak β-hemolysis (clearing of the agar) by the white colony in the upper right (arrow). White growth with β-hemolysis is characteristic of *Staphylococcus aureus*. For information about hemolytic reactions on blood agar plates, see Exercise 5-25.

References

Claus, G. William. Chap. 14 in *Understanding Microbes—A Laboratory Textbook for Microbiology*. New York: W. H. Freeman and Co., 1989.

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Chap. 6 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, England: Butterworth-Heineman, 1995.

Tille, Patricia M. Pages 92–93 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

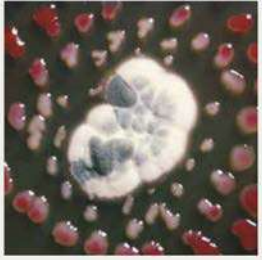
Winn, Washington C. et al. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-2

Colony Morphology

OBSERVATIONS AND INTERPRETATIONS

1 Use the terms in Figure 2.3 and the text to describe and carefully sketch representative colonies (including side views) on your plates, including those from Exercise 2-1 if not already done. Use a colony counter if necessary. Measure colony diameters, and include them with your descriptions along with incubation times and temperatures, and culture sources.

Organism/Plate	Colony Description and Sketch

QUESTIONS

1 *A description of colony morphology provides important information about an organism. What other colony features should you include?*

2

2 *Three critical aspects of a description of bacterial growth are colony size, color, and shape. At least three other important factors—not descriptions of the organism itself—typically are included when describing bacterial growth. What are they and why are they important?*

EXERCISE
2-3

Growth Patterns on Slants

Theory

Agar slants are useful primarily as media for cultivation and maintenance of stock cultures. Organisms cultivated on slants, however, do display a variety of growth characteristics. We offer these more as an item of interest than one of diagnostic value.

Most of the organisms you will see in this class produce **filiform** growth (dense and opaque with a smooth edge. All of the organisms in Figure 2.34 are filiform and **pigmented**. Growth texture also may vary (Fig. 2.35). Most species in the genus *Mycobacterium* produce **friable** (crusty) growth. Some motile organisms produce growth with a **spreading edge** (e.g., *Alcaligenes faecalis*). Still others produce **translucent** or **transparent** growth (e.g., *Lactobacillus plantarum*).

As with colony descriptions, the edge (margin) of growth on a slant may be entire, lobed, or irregular. Of the photographs shown, only *M. phlei* in Figure 2.35 has a margin other than entire. It is irregular. What appears to be "waviness" in the margins of the other tubes (e.g., Fig. 2.34) is due to the pattern of inoculation by the microbiologist. That is, the loop was moved side-to-side as it was being removed from the tube. Careful examination shows that irregularities in the margins of growth are artifacts of inoculation.

Growth patterns of virtually all microorganisms can be influenced by environmental factors. Two important factors influencing bacterial growth are incubation time and temperature. Figure 2.36 illustrates the effect of temperature on pigment production in *Serratia marcescens*. See "Environmental Factors Affecting Microbial Growth" on page 83 for further discussion.

2.35 Growth Texture on Slants ♦ From left to right, *Bacillus* sp. (flat, dry), *Alcaligenes faecalis* (spreading edge), *Mycobacterium phlei* (crusty/friable), *Lactobacillus plantarum* (transparent, barely visible).

2.34 Pigment Production on Slants ♦ From left to right: *Staphylococcus epidermidis* (white), *Pseudomonas aeruginosa* (green), *Chromobacterium violaceum* (violet), *Serratia marcescens* (red/orange), *Kocuria rosea* (rose), *Micrococcus luteus* (yellow). All of these organisms have an entire (smooth) edge. The waviness you see is due to the inoculation pattern.

2.36 Influence of Temperature on Pigment Production in *Serratia marcescens* ♦ *Serratia marcescens* was grown for 48 hours on tryptic soy agar slants at five different temperatures. From left to right: 25°C, 30°C, 33°C, 35°C, and 37°C. A difference of 2°C makes the difference between being pigmented or not!

Application

Although not definitive by themselves, growth characteristics on slants can provide useful information when attempting to identify an organism.

2

In This Exercise

In Exercise 2-2, you examined colony growth on agar plates. Today you will be looking at the growth patterns of up to seven organisms on prepared agar slants. If your instructor had you save your plates from Exercise 2-2, you will compare the growth patterns on the two different types of media.

✓ Materials

Per Student Group

- ☐ Tryptic soy agar or brain-heart infusion agar slant cultures of:
  - *Bacillus subtilis*
  - *Corynebacterium xerosis*
  - *Kocuria rosea*
  - *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - *Micrococcus luteus*
- ☐ (Optional)
  - *Mycobacterium smegmatis* (BSL-2)
  - *Proteus mirabilis* (BSL-2)
- ☐ Uninoculated tube of the medium used for cultures.

Procedure

1. Examine the slants and describe the different growth patterns on the data sheet, page 77. Include a sketch of a representative portion of each.
2. (Optional) Compare the slant growth to the colonies on the corresponding agar plates from Exercise 2-2.
3. (Optional) Save the tubes for comparison with broths in Exercise 2-4.

Reference

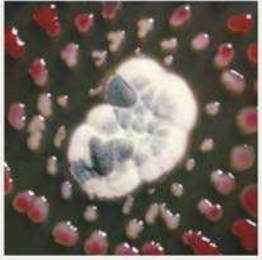
Claus, G. William. Chap. 17 in *Understanding Microbes—A Laboratory Textbook for Microbiology*. New York: W. H. Freeman and Co., 1989.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-3

Growth Patterns on Slants

OBSERVATIONS AND INTERPRETATIONS

1 In the table below, describe the growth on your slants, including shape, margin, texture, and color. Draw a picture of a short segment, if necessary, and include other information about the conditions of incubation, such as time, temperature, and the medium used.

Organism	Colony Description and Sketch
Control	

QUESTIONS

1 *Match the following:*

_____ *Filiform*	1. *Produces colored growth*
_____ *Spreading edge*	2. *Smooth texture with solid edge*
_____ *Transparent*	3. *Solid growth seeming to radiate outward*
_____ *Friable*	4. *Almost invisible, or easy to see light through*
_____ *Pigmented*	5. *Rough texture with crusty appearance*

2 *List some reasons why growth characteristics are more useful on agar plates than on agar slants.*

3 *Why are agar slants better suited than agar plates to maintain stock cultures?*

4 *Suggest possible reasons for how temperature affects pigment production (as in Fig. 2.36).*

EXERCISE
2-4

Growth Patterns in Broth

Theory

Microorganisms cultivated in broth display a variety of growth characteristics. Some organisms float on top of the medium and produce a type of surface membrane called a **pellicle.** Others sink to the bottom as **sediment.** Some bacteria produce **uniform fine turbidity,** and others appear to clump in what is called **flocculent** growth. Others show pigmentation. Refer to Figures 2.37 through 2.39.

Application

Bacterial genera, and frequently individual species within a genus, demonstrate characteristic growth patterns in broth that provide useful information when attempting to identify an organism.

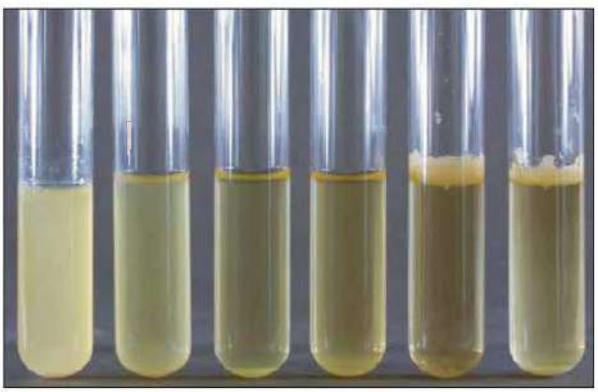
In This Exercise

Today, you will be examining the growth characteristics of six different bacteria in broth. If you were instructed to save plates or slants from previous exercises, you will compare the growth patterns on the different types of media.

Materials

Per Student Group

- ☐ One sterile uninoculated brain–heart infusion (BHI) broth control
- ☐ BHI broth cultures of:
  - *Bacillus subtilis*
  - *Corynebacterium xerosi*
  - *Kocuria rosea*
  - *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - *Micrococcus luteus*
- ☐ (Optional)
  - *Mycobacterium smegmatis* (BSL-2)
  - *Proteus mirabilis* (BSL-2)
- ☐ Your slants from Exercise 2-3



2.37 Growth Patterns in Broth ♦ From left to right in pairs (of similar species): Uniform fine turbidity–UFT: *Enterobacter aerogenes* and *Citrobacter diversus*—motile members of *Enterobacteriaceae*; Sediment: *Enterococcus faecalis* and *Staphylococcus aureus*—nonmotile Gram-positive cocci; Pellicle–floating on surface: *Mycobacterium phlei* and *Mycobacterium smegmatis* (relatives of *Mycobacterium tuberculosis*)—nonmotile with a waxy cell wall. Other organisms (e.g., *Halobacterium salinarum*) form a pellicle due to the presence of gas vesicles that give them buoyancy.

2.38 Flocculence in Broth ♦ This is a *Streptococcus* species from a throat culture demonstrating flocculence in Todd-Hewitt broth.

2.39 Pigmentation in Broth ♦ *Rhodospirillum rubrum* has a red color due to carotenoid pigments. It grows as a photoheterotroph in the presence of light and the absence of oxygen.

Procedure

Examine the tubes and compare them with the control. Describe the different growth patterns in the table on the data sheet, page 81.

2

References

Claus, G. William. Chap. 17 in *Understanding Microbes—A Laboratory Textbook for Microbiology*. New York: W. H. Freeman and Co., 1989.

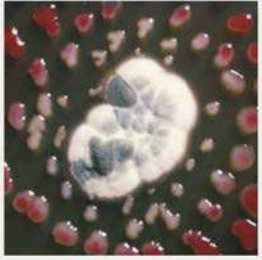
Grant, William D. Page 301 in Genus I. *Halobacterium* in *Bergey's Manual of Systematic Bacteriology*, 2nd ed. Vol. 1, The Archaea and The Deeply Branching and Phototrophic Bacteria. David R. Boone and Richard W. Castenholz, eds. New York: Springer, 2001.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-4

Growth Patterns in Broth

OBSERVATIONS AND INTERPRETATIONS

1 Examine the tubes and enter the descriptions in the table below. Draw a picture if necessary, and include other information about the conditions of incubation, such as time, temperature, and the medium used.

Organism	Sketch and Description of Growth in Broth
Control	

QUESTIONS

1 *Match the following:*

_____ *Flocculent*	a. *Evenly cloudy throughout*
_____ *Sediment*	b. *Growth at top around the edge*
_____ *Ring*	c. *Growth on the bottom*
_____ *Pellicle*	d. *Membrane at the top*
_____ *Uniform fine turbidity*	e. *Suspended chunks or pieces*

2 *What factors besides physical growth characteristics are important when recording data about an organism? Why?*

Environmental Factors Affecting Microbial Growth

All organisms rely on their environment to provide them with the resources and conditions necessary for their growth. That's actually another way of saying, "Organisms are adapted to their environment." However, not all organisms are equally dependent on their environment, and not all are equally finicky about their environmental conditions. With the disclaimer that absence of *any* nutritional requirement is enough to prevent microbial growth, three elements are of major importance. These are carbon, nitrogen, and oxygen. As you go through this lab course you will be using media and incubation conditions that attempt to mimic the organisms' natural habitats, if not perfectly, then well enough to support their growth. Be sure to examine the media recipes in each exercise and identify the source(s) of these elements. It will help you understand the exercise as well as getting a better understanding of the organisms themselves.

Probably the most obvious environmental need organisms have is colloquially referred to as "food." However, the concept of "food" is more complex than you might think, because organisms need energy *and* they need a carbon source, along with numerous other elements (recall the acronym CHOPKNSCaFe—read as "C. HOPK(i)NS café—for starters). But, we will focus on energy and carbon right now. Some organisms get energy from chemicals and are classified as **chemotrophs**, whereas others get their energy from light and are called **phototrophs**—and most people wouldn't consider light as "food." Organisms able to survive with carbon dioxide (CO_2) as the only carbon source are classified as autotrophs, and again most people wouldn't consider CO_2 as "food," yet it is a necessary resource for autotrophs. Organisms requiring organic carbon are classified as **heterotrophs**. For many organisms (called **chemoheterotrophs**) the same organic molecule(s) provide both carbon and energy. (If you are feeling self-conscious about now, you should: you are a chemoheterotroph!)

In Exercise 2-5 you will be examining the role of medium composition in determining what organism(s) it can support and how effectively it can do so. You will also be examining how finicky different organisms can be with respect to dependence on their environment. Optimal growth conditions, as might be expected, result in faster growth and greater cell density (as evidenced by greater turbidity) than do less than optimal conditions. Understand that growth rate, as it is used in this manual, is synonymous with reproductive rate, and while inherent growth rates may differ for each organism, we can legitimately compare growth rates of the same organism in different media. Growth rate comparisons between different organisms in the *same* medium requires the assumption that the inherent growth rates are the same or similar, which we will make with the understanding that it may not be true. ◆

EXERCISE 2-5

Evaluation of Media

Theory

Living things are composed of compounds from four biochemical families:

- proteins,
- carbohydrates,
- lipids, and
- nucleic acids.

Even though all organisms share this fundamental biochemical composition, they differ greatly in their ability to make these molecules. Some are capable of making them out of the simple carbon compound carbon dioxide (and a nitrogen source). These organisms, called autotrophs, require the least "assistance" from the environment to grow. The remaining organisms, called heterotrophs, require preformed organic compounds from the environment.

Some heterotrophs are metabolically flexible and require only a few simple organic compounds from which to make all their biochemicals. Others require a greater portion of their organic compounds from the environment. An organism that relies heavily on the environment to supply ready-made organic compounds is referred to as **fastidious**. Heterotrophs vary greatly in their dependence on the environment to supply organic

compounds and, as such, range from highly fastidious to **nonfastidious**. Autotrophs are less fastidious than the most nonfastidious heterotrophs.

Successful cultivation of a microbe in the laboratory requires an ability to satisfy its nutritional needs. The absence of a single required chemical resource prevents its growth. In general, the more fastidious the organism, the more ingredients a medium must have. **Undefined media** (also known as **complex media**) are composed of extracts from, or digests of, plant or animal matter and are usually quite rich in nutrients. Even though the exact composition of the medium and the amount of each ingredient are unknown, undefined media are useful in growing the greatest variety of culturable microbes. A **defined medium** (or **chemically defined medium**) is one in which the amount and identity of every ingredient are known, which means it must be formulated from purified chemicals. As a result, defined media typically support a narrower range of organisms and are usually used with the growth of a particular organism or group of organisms in mind.

Application

The ability of a microbiologist to cultivate a microorganism requires some knowledge of its metabolic needs. One quick way to make this determination is to transfer it to a variety of media containing different nutritional components and observe how well it grows.

In This Exercise

Today you will evaluate the ability of three media—brain–heart infusion broth, nutrient broth, and glucose salts medium—to support bacterial growth. You will do this by visually comparing the density of growth (turbidity) of organisms in the various broths. It is important to make your inoculations as uniform as possible, so you will be adding the inocula with sterile transfer pipettes.

✓ Materials

Per Student Group

- ☐ Five tubes each of:
  - Brain–heart infusion broth
  - Nutrient broth
  - Glucose salts broth
- ☐ BHI broth cultures of:
  - *Escherichia coli*
  - *Lactococcus lactis*
  - *Moraxella catarrhalis* (BSL-2)
  - *Staphylococcus epidermidis*
- ☐ Four sterile transfer pipettes

Medium Recipes

Nutrient Broth

Beef extract	3.0 g
Peptone	5.0 g
Distilled or deionized water	1.0 L

Brain–Heart Infusion Broth

Calf brains, infusion from 200 g	7.7 g
Beef heart, infusion from 250 g	9.8 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Distilled or deionized water	1.0 L

Glucose Salts Broth

Glucose	5.0 g
Sodium chloride	5.0 g
Magnesium sulfate	0.2 g
Ammonium dihydrogen phosphate	1.0 g
Dipotassium phosphate	1.0 g
Distilled or deionized water	1.0 L

Procedure

Lab One

1. Obtain five tubes of each medium (a total of 15 tubes) and label each with your name, the date, and the medium. Label four sets of the tubes with the names of the organisms, one name on each set of tubes; label the fifth set of tubes "uninoculated."
2. Each student should choose one or two cultures to inoculate from (whatever divides the work load evenly). Mix them thoroughly, but be careful not to splash into the cap. **Use BSL-2 precautions when transferring *Moraxella catarrhalis*.**
3. Inoculate one tube of each medium with one drop of the organism (be consistent) using a sterile transfer pipette. You may fill the pipette with about 1 mL of broth and deliver the drops with the same pipette to the tubes without flaming them. In your group, leave one of each tube uninoculated.

   Caution!

   - Insert the pipette well into the tube (but not touching the glass or the medium) before delivering the inoculum.
   - Be careful not to spatter as you deliver the drop(s) to each tube, or accidentally drip organism between tubes. Clean up the spill if you do.
   - If you don't have enough organism left in the pipette to inoculate a tube, get more organism. You must not empty ("blow-out") the pipette because you will create aerosols.
   - Properly dispose of the pipette when finished.
4. Incubate all tubes (including the uninoculated ones) at 35 ± 2°C for 24–48 hours.

Lab Two

1. Clean the outside of all tubes with a tissue, and place them in a test tube rack organized into groups by organism in one direction and by medium in the other.
2. Mix all tubes well and examine them for turbidity. Be careful not to splash broth into the caps. Score relative amounts of growth using "0" for no growth, and "1," "2," and "3" for successively greater degrees of growth. Choose the tube (regardless of medium) with the most turbidity to act as a "3." What will you use to act as "0"?

   _____

   _____

   _____
3. Record your results on the data sheet, page 87. (***Note:*** Results may vary. Record what you see, not what you expect.)

References

Delost, Maria Dannessa. Page 144 in *Introduction to Diagnostic Microbiology: A Text and Workbook*. St. Louis, MO: Mosby, 1997.

Tille, Patricia M. Chap. 7 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

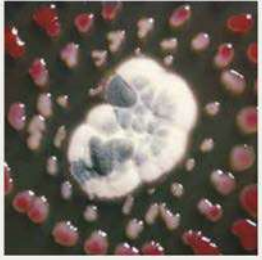
Zimbro, Mary Jo and David A. Power, eds. *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-5

Evaluation of Media

OBSERVATIONS AND INTERPRETATIONS

1 In the table below, score the relative amount of growth in each tube. Compare the five tubes of each medium with each other, as well as the amount of growth for each organism in the various media. Use "0" for no growth, "3" for abundant growth, and "1" and "2" for degrees of growth in between. Use the most turbid broth as your "3."

Specimen	Nutrient Broth	Brain-Heart Infusion Broth	Glucose Salts Broth	Interpretation (relative fastidiousness)
Uninoculated control				

2 In the table below, identify the carbon and nitrogen source(s) in each medium and then characterize each as "defined" or "undefined" ("complex"). If undefined, list the ingredient(s) that make it undefined. Finally, use your results to characterize each medium based on its ability to support growth of a wide range of organisms (poor, fair, good).

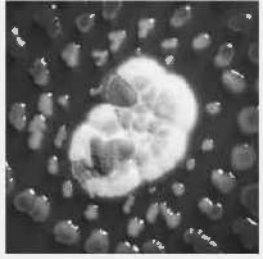
Medium Description	Nutrient Broth	Brain-Heart Infusion Broth	Glucose Salts Broth
Carbon source(s)			
Nitrogen source(s)			
Defined/undefined			
Ingredient(s) making it "undefined"			
Ability to support a wide range of organisms (poor, fair, good)			

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-5

(continued)

QUESTIONS

1 *Why is it recommended that you use the broth (regardless of medium) with the most growth to serve as the "standard" for scoring a "3"?*

2 *Why is it important to use the uninoculated controls for each medium to serve as the "standard" for scoring a "0" in their respective tubes?*

3 *What does comparing growth of a given organism in the three media tell you? (That is, are you gaining information primarily about the organism or about the medium?)*

4 *What does comparing growth of the four organisms in a given medium tell you? (Again, are you gaining information primarily about the organism or about the medium?)*

5 *Suppose after performing this experiment, you examine the growth of two different organisms in Medium X. Both show growth and Organism #1 has produced less turbidity than Organism #2. Explain how it could be possible that Organism #1's needs are actually* better *supplied by the medium than Organism #2's, yet there is less turbidity.*

6 *Evaluate the media.*

a. *Which medium supports growth of the widest range of organisms?*

b. *Which medium supports the fewest organisms?*

c. *Is there a correlation between your answers to questions 6a and 6b and the terms* defined medium *and* undefined medium*? If so, what is it? If not, attempt to explain the relationship you observe.*

7 *Evaluate the organism.*

a. *Which organism appears to be most fastidious? How can you tell?*

b. *Which organism appears to be least fastidious? How can you tell?*

8 *What is the biochemical basis for the spectrum of fastidiousness seen in the microbial world? (That is, why are some organisms fastidious and others are nonfastidious?)*

Aerotolerance

Most microorganisms can survive within a range of environmental conditions, but not surprisingly, tend to produce growth with the greatest density in the areas where conditions are most favorable. One important resource influencing microbial growth is oxygen (O_2)[1]. Some organisms require oxygen for their metabolic needs (e.g., in aerobic respiration). Remarkably (from our perspective) other organisms don't use it and cannot even survive in its presence! Still other organisms don't use it and are not affected by it at all. This ability or inability to live in the presence of oxygen is called **aerotolerance**.

Most growth media are sterilized in an autoclave during preparation. This process not only kills unwanted microbes, but removes most of the free oxygen from the medium as well. After the medium is removed from the autoclave and allowed to cool, oxygen begins to diffuse back in from the air (which is approximately 21% O_2). In tubed media (both liquid and solid) this process creates a gradient of oxygen concentrations, ranging from **aerobic** at the top, nearest the source of oxygen, to **anaerobic** at the bottom. Because of microorganisms' natural tendency to proliferate where the oxygen concentration best suits their metabolic needs, differing degrees of population density will develop in the medium over time that can be used to visually examine their aerotolerance.

Obligate (strict) aerobes, organisms that require oxygen for aerobic respiration, grow at the top where oxygen is most plentiful. **Facultative anaerobes** grow in the presence *or* absence of oxygen. When oxygen is available, they respire aerobically. When oxygen is not available, they either respire anaerobically (reducing sulfur or nitrate instead of oxygen) or ferment an available substrate. Refer to Appendix A and Section Five for more information on anaerobic respiration and fermentation. Where an oxygen gradient exists, facultative anaerobes grow throughout the medium but appear denser near the top because of the higher ATP yield from aerobic respiration compared to fermentation or anaerobic respiration. **Aerotolerant anaerobes,** organisms that don't require oxygen and are not adversely affected by it, live uniformly throughout the medium. Aerotolerant anaerobes are fermentative even in the presence of free oxygen. **Microaerophiles,** as the name suggests, survive only in environments containing lower than atmospheric levels of oxygen. Some microaerophiles called **capnophiles** can survive only if carbon dioxide levels are elevated. Microaerophiles will be seen somewhere near the middle or upper middle region of the medium. Finally, **obligate (strict) anaerobes** are organisms for which even small amounts of oxygen are lethal and, therefore, will be seen only in the lower regions of the medium, depending on how far into the medium oxygen has diffused. ◆

[1] Most biochemicals and many inorganic compounds contain oxygen, so all organisms require oxygen as part of other molecules. The use of the term *oxygen* in most microbiological contexts refers to molecular oxygen.

EXERCISE 2-6

Agar Deep Stabs

Theory

Agar deep stabs are prepared with tryptic soy agar (TSA) enriched with yeast extract to promote growth of a broad range of organisms. As stated in the introduction to this section, oxygen, which is removed from the medium during preparation and autoclaving, immediately begins to diffuse back into the medium as the agar cools and solidifies. This process creates an oxygen gradient in the medium, ranging from aerobic at the top to anaerobic at the bottom.

Agar deeps are prepared with 10 mL of medium instead of the customary 7 mL used in slants. This extra depth ensures (if inoculated soon after preparation) that the bottom portion of the medium is anaerobic. The agar is stab-inoculated with an inoculating needle to introduce as little air as possible. The location of growth that develops indicates the organism's aerotolerance (Fig. 2.40).

Application

This test is a good visual indicator of oxygen tolerance in microorganisms.

2.40 Agar Deep Stab Tubes ♦ Shown are three agar deep tubes inoculated with different organisms. When agar is autoclaved, air is removed. Once the agar cools and solidifies, air diffuses back into the agar of agar deep tubes (at least over the short term) and a concentration gradient is created. Organisms are then stab inoculated into the agar with an inoculating needle. After incubation, their aerotolerance category can be determined by where they have grown within the concentration gradient. If growth is only seen in the upper couple of centimeters, the organism is an obligate aerobe (left). If the organism grows throughout the depth of the stab and better near the surface, it is a facultative anaerobe (middle tube). Lastly, if the organism only grows in the deeper parts of the agar, it is an obligate anaerobe (right tube).

In This Exercise

Today you will stab-inoculate three agar tubes to determine the aerotolerance categories of three bacteria.

✓ Materials

Per Student Group

- ☐ Four agar deep-stab tubes
- ☐ Inoculating needle
- ☐ Fresh cultures of:
  - *Alcaligenes faecalis* subsp. *faecalis*
  - *Clostridium butyricum*
  - *Staphylococcus epidermidis*

Medium Recipe

Enriched TSA

• Tryptone	15.0 g
• Soytone	5.0 g
• Sodium chloride	5.0 g
• Yeast extract	5.0 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L

Procedure

Lab One

1. Obtain four agar deep-stab tubes and label each with your name, the date, and the medium. Label three of the tubes with the name of the organisms, one name on each tube; label the fourth tube "sterile stab."
2. With a heavy inoculum on your inoculating needle, carefully stab the agar tubes with the test organisms. Refer to Appendix B for the proper stab technique. (***Note:*** To minimize the introduction of unwanted oxygen to the medium, try to avoid lateral motion of the needle during inoculation.)
3. Stab the fourth tube with your sterile needle.
4. Incubate the tubes at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Examine the tubes and determine the aerotolerance category of each organism.
2. Record your results on the data sheet, page 93.

References

Koneman, Elmer W., Stephen D. Allen, William M. Janda, Paul C. Schreckenberger, and Washington C. Winn, Jr. Chap. 14 in *Color Atlas and Textbook of Diagnostic Microbiology*, 5th ed. Philadelphia: J. B. Lippincott Co., 1997.

Ryan, Kenneth J. and C. George Ray. Pages 64–65 in *Sherris Medical Microbiology*, 6th ed. New York: McGraw-Hill, 2014.

Tille, Patricia M. Chap. 7 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

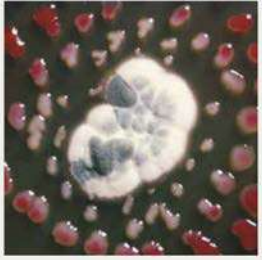
Willey, Joanne, Linda Sherwood, and Chris Woolverton. Chap. 7 in *Prescott's Microbiology*, 7th ed. New York: McGraw-Hill, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-6

Agar Deep Stabs

OBSERVATIONS AND INTERPRETATIONS

1 Examine all tubes and enter a description and sketch of each in the table below.

2 Categorize each organism with respect to its aerotolerance group based on the location of growth in the medium.

Organism	Location and Relative Amount of Growth in Medium		Aerotolerance Category
Sterile stab			NA
	Aerobic zone	*Sketch*	
	Anaerobic zone		
	Aerobic zone	*Sketch*	
	Anaerobic zone		
	Aerobic zone	*Sketch*	
	Anaerobic zone		

QUESTIONS

1 *Why is it important to use this medium soon after preparation?*

2 *When inoculating agar deep stabs, why do you have to insert and remove the needle along the same stab line?*

3 *Suppose that after incubation you find a tube that indicates uniform growth through the agar.*

a. *Can the aerotolerance category of the organism be determined? Explain.*

b. *With which aerotolerance group are you most likely to confuse this tube and how will you distinguish between them?*

4 *If you inoculate an organism known to chemically reduce sulfur (as done by organisms capable of anaerobic respiration), where would you expect to see its growth in the test medium used today? Could it be seen in more than one zone? Explain.*

5 *If you have no growth in the stab after 24 hours' incubation, what are some possible explanations?*

EXERCISE 2-7

Fluid Thioglycollate Broth

Theory

Fluid thioglycollate broth (FTB) is prepared as a basic medium (as used in this exercise) or with a variety of supplements, depending on the specific needs of the organisms being cultivated. As such, this medium is appropriate for a broad variety of aerobic and anaerobic, fastidious and nonfastidious organisms. It is particularly well adapted for cultivation of strict anaerobes and microaerophiles.

Key components of the medium are yeast extract, pancreatic digest of casein, dextrose, sodium thioglycollate, L-cystine, and resazurin. Yeast extract and pancreatic digest of casein provide nutrients; sodium thioglycollate and L-cystine reduce oxygen to water; and resazurin (pink when oxidized, colorless when reduced) acts as an indicator of O_2. A small amount of agar is included to slow oxygen diffusion.

As mentioned in the Aerotolerance sidebar on page 91, oxygen removed during autoclaving will diffuse back into the medium as the tubes cool to room temperature. This produces a gradient of concentrations from aerobic (comparable to air) at the top to anaerobic at the bottom. Thus, fresh media will appear clear to straw-colored with a pink region at the top where the dye has become oxidized (Fig. 2.41). Figure 2.42 shows some basic bacterial growth patterns in the medium as influenced by the oxygen gradient.

Application

Fluid thioglycollate broth is a liquid medium that can be modified to promote growth of a wide variety of fastidious to nonfastidious microorganisms. It can be used to illustrate microbial growth representing all levels of oxygen tolerance; however, it generally is associated with the cultivation of anaerobic and microaerophilic bacteria.

In This Exercise

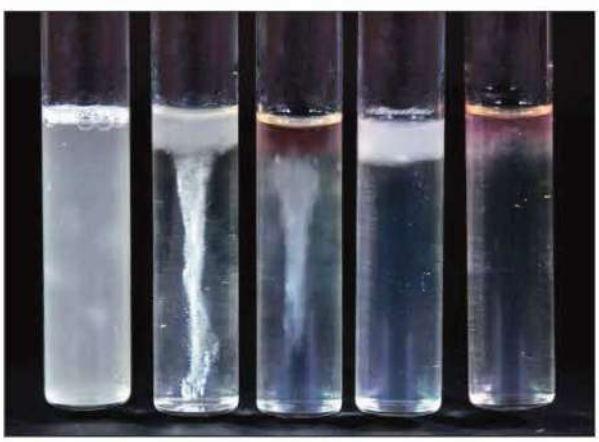
Today you will be inoculating fluid thioglycollate broth to determine the aerotolerance categories of three bacteria. Your instructor may have additional organisms or may have you collect environmental samples to test.

✓ Materials

Per Student Group

- ☐ Four fluid thioglycollate broth (FTB) tubes
- ☐ Fresh cultures of:
  - *Alcaligenes faecalis* subsp. *faecalis*
  - *Clostridium butyricum*
  - *Staphylococcus epidermidis*

2.41 Aerobic Zone in Thioglycollate Broth ♦ Note the pink region in the top (oxidized) portion of the broth resulting from the indicator resazurin. In the bottom (reduced) portion, the dye is colorless and the medium is its typical straw color.



2.42 Growth Patterns in Thioglycollate Broth ♦ Growth patterns of a variety of organisms are shown in these fluid thioglycollate broths. Pictured from left to right are: aerotolerant anaerobe, facultative anaerobe, strict anaerobe, strict aerobe, and microaerophile. Compare these tubes with the uninoculated broth in Figure 2.41.

Medium Recipe

Fluid Thioglycollate Broth

• Yeast extract	5.0 g
• Pancreatic digest of casein	15.0 g
• Dextrose	5.5 g
• Sodium chloride	2.5 g
• Sodium thioglycollate	0.5 g
• L-cystine	0.5 g
• Agar	0.75 g
• Resazurin	0.001 g
• Distilled or deionized water	1.0 L

Procedure

Lab One

1. Obtain four fluid thioglycollate tubes and label them with your name, the date, and the medium. Label three of the tubes with the name of the organism, one name on each tube; label the fourth tube "uninoculated."
2. Using your loop, inoculate three broths with the organisms provided. (*Note:* When inoculating thioglycollate broth, it helps to dip the loop all the way to the bottom of the tube and gently mix the broth with the loop as you remove it. Finish mixing by gently rolling the tube between your hands.) Do not inoculate the fourth tube.
3. Incubate the tubes at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Check the control tube for growth to assure sterility of the medium. Note any changes that may have occurred as a result of incubation, especially in the colored region at the surface.
2. Using the control as a comparison, examine and note the location of the growth in all tubes.
3. Enter your observations and interpretations in the table provided on the data sheet, page 97.

References

Allen, Stephen D., Christopher L. Emery, and David M. Lyerly. Chap. 54 in *Manual of Clinical Microbiology*, 8th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Michael A. Pfaller, and Robert H. Yolken, eds. Washington, DC: ASM Press, American Society for Microbiology, 2003.

Atlas, Ronald M. and James W. Snyder. Page 299 in *Manual of Clinical Microbiology*, 10th ed., James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Ryan, Kenneth J. and C. George Ray. Pages 64–65 in *Sherris Medical Microbiology*, 6th ed. New York: McGraw-Hill, 2014.

Tille, Patricia M. Pages 85 and 87–88 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Washington C. et al. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

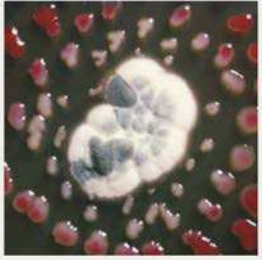
Zimbro, Mary Jo and David A. Power, Eds. *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Company, 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-7

Fluid Thioglycollate Broth

OBSERVATIONS AND INTERPRETATIONS

1 Draw a sketch of each culture showing the location of growth. Indicate the amount of growth in the broth using the following system:

"O" = no growth "3" = abundant growth "1" and "2" = degrees of growth in between

Then, place each organism into an aerotolerance category.

Organism	Location of Growth in Medium	Aerotolerance Category
Control		NA

QUESTIONS

1 *Consider aerotolerance categories and their growth in FTB.*

a. *Where would you expect to see growth of a strict aerobe?*

b. *Anaerobe?*

c. *Microaerophile?*

d. *Facultative anaerobe?*

e. *Aerotolerant anaerobe?*

2 *Consider resazurin dye. Considering the major application of this medium, which is more desirable, a thick colored or a thin colored band?*

a. *What purpose does the dye serve in FTB?*

b. *Where in the broth column is resazurin dye located?*

c. *Why is the broth pink at its surface?*

d. *Considering the primary application of FTB, which is more desirable, a thick or a thin colored band at the surface?*

3 *Consider medium freshness.*

a. *Why is it important that this medium be fresh?*

b. *Which type of organism (obligate aerobe, obligate anaerobe, microaerophile, facultative anaerobe, aerotolerant anaerobe) would most likely be affected negatively by the use of old media?*

c. *Which would most likely be affected positively by the use of old media?*

d. *Which would be least affected by old media?*

Anaerobic Jar

EXERCISE 2-8

Theory

The GasPak® Anaerobic System by BBL™ is a plastic jar in which to create anaerobic, microaerophilic, or CO_2-enriched conditions, depending on the specific needs of the bacteria being cultivated. The components required for anaerobic growth include a chemical gas generator packet (envelope) containing sodium borohydride and sodium bicarbonate, and a paper indicator strip saturated with methylene blue or resazurin to confirm the absence of oxygen (Fig. 2.43). Both methylene blue and resazurin are colorless when reduced, but are blue and pink, respectively, when oxidized. Also included in the packet is a small amount of palladium to act as a catalyst for the reaction that will produce the necessary conditions inside the jar.

After the inoculated media (typically agar plates) are placed inside the jar, the opened gas generator envelope is placed inside along with the anaerobic indicator strip. Water is added to the envelope and the jar lid immediately is fastened down. The sodium borohydride and sodium bicarbonate in the envelope react with the water to produce hydrogen and carbon dioxide gases. The palladium catalyzes a reaction between the hydrogen and free oxygen in the jar to produce water, as shown in Figure 2.44. Removal of free oxygen produces anaerobic conditions in the jar within approximately an hour, as evidenced by a white indicator strip and moisture on the inside of the jar.

Oxoid AnaeroGen Atmospheric Generation Systems use an alternative method of generating anaerobic and microaerophilic conditions. These chemically reduce

2.43 The Anaerobic Jar ◆ Note the white methylene blue strip and the open packet, which has discharged H_2 and CO_2 gases. The palladium, contained in the packet, catalyzes the conversion of H_2 and O_2 as shown in Figure 2.44.

$$2H_2 + O_2 \xrightarrow{Pd} 2H_2O$$

2.44 Conversion of H_2 and O_2 to Water Using a Palladium Catalyst ◆ The gas generator packet produces H_2 gas, which subsequently reacts with O_2 to make water, thus reducing the O_2 concentration inside the jar.

oxygen within the jar to less than 1% in 30 minutes and raise CO_2 levels to 9%–13% in 30 minutes. The advantage of these systems is they don't require the addition of water or the palladium catalyst, and don't produce H_2 gas.

Figure 2.45 illustrates some typical growth patterns under aerobic and anaerobic conditions.

Application

This procedure provides a means of conveniently cultivating anaerobic and microaerophilic bacteria without expensive equipment.

In This Exercise

Today you will use the jar to experience a common way to grow anaerobes (or microaerophiles), but you will also take advantage of the opportunity to demonstrate aerotolerance categories of the organisms grown. To do the latter, you will inoculate two nutrient agar plates with three organisms (your instructor may have additional organisms for you to test) and incubate one of the plates inside the jar and the other outside the jar. Both plates will be incubated at 35 ± 2°C for 24 to 48 hours. Following incubation, growth on the two plates will be examined and compared to determine aerotolerance.

✓ Materials

Per Class

- ☐ One anaerobic jar with gas generator packet[1,2]

Per Student Group

- ☐ Two nutrient agar plates
- ☐ Fresh broth cultures of:
  - *Alcaligenes faecalis* subsp. *faecalis*
  - *Clostridium butyricum*
  - *Staphylococcus epidermidis*

[1] Available from Becton Dickinson Microbiology Systems, Sparks, MD http://www.bd.com

[2] Available from Thermo Fisher Scientific, Oxoid Microbiology Products, Waltham, MA, http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=AN0035&cc=UK&lang=EN

2.45 Plates Incubated Inside and Outside the Anaerobic Jar ♦ Both nutrient agar plates were inoculated with the same facultative anaerobe (top), strict aerobe (right), and strict anaerobe (left). The plate on the left was incubated outside the jar, and the plate on the right was incubated inside the jar. Note the relative amounts of growth of the three organisms.

Procedure

Lab One

1. Obtain two nutrient agar plates. Using a marking pen, divide the bottom of each plate into three sectors (or more, if your instructor has additional organisms for you to use).
2. Label each plate with your name, the date, and organism by sector.
3. Using your loop, inoculate corresponding sectors of both plates with the organisms provided. (*Note:* Inoculate with single streaks about one centimeter long.) Tape the lids in place.
4. Place one plate in the anaerobic jar in an inverted position.
5. When all groups have placed their plates in the jar, discharge the packet as follows:

If using a BD system,

- a Stick the methylene blue strip on the wall of the jar.
- b Open the packet and add 10 mL of distilled water.
- c Place the open packet in the jar with the label facing inward.
- d Immediately close the jar.
- e Examine for condensation within about 30 minutes. If none appears, start over with a new packet.

If using an Oxoid system,

- a Stick the resazurin indicator strip on the wall of the jar.
- b Open the foil packet and remove the sachet. Place the sachet in the jar.
- c Close the jar within one minute.

6. Place the second plate and the anaerobic jar in the 35 ± 2°C incubator for 24 to 48 hours. Be sure the plate is inverted.

Lab Two

1. Examine and compare the growth on the plates. (*Note:* Density of growth will be the most useful basis for comparison.)
2. Properly dispose of the packet in laboratory waste. The Oxoid packet may still be warm to the touch. Let it cool before disposing of it.
3. Record your results and interpretations in the table provided on the data sheet, page 101.

References

Ryan, Kenneth J. and C. George Ray. Pages 64–65 in *Sherris Medical Microbiology*, 6th ed. New York: McGraw-Hill, 2014.

Thermo Fisher Scientific, Oxoid Microbiology Products, Waltham, MA, http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=AN0035&cc=UK&lang=EN

Tille, Patricia M. Pages 460–464 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Washington C. et al. 2006. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

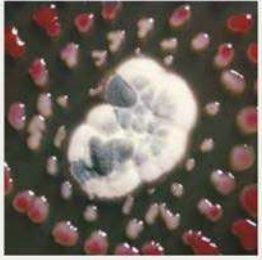
Zimbro, Mary Jo, and David A. Power, eds. *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Company, 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-8

Anaerobic Jar

OBSERVATIONS AND INTERPRETATIONS

1 Indicate the amount of growth on each plate.

"0" = no growth "3" = dense growth "1" and "2" = degrees of growth in between

Organism	Growth on Aerobic Plate	Growth on Anaerobic Plate	Aerotolerance Category

QUESTIONS

1 *Suppose that after incubation you examine the jar before opening it and see that the methylene blue strip is blue (or the resazurin strip is pink).*

a. *What would you guess the internal environment to be—aerobic or anaerobic?*

b. *How would you expect the growth on the plates inside the jar to differ from the plates incubated outside the jar?*

2 *Based on your results, which of the three organisms would be most affected by the conditions described in question 1?*

3 *An alternative to the anaerobic jar is a candle jar, in which a candle is placed in the jar, lit, and the lid closed to enable the flame to use the available oxygen. Typically, in this system, not all of the oxygen is used. Which types of organisms would most likely benefit from this environment?*

4 *Considering the gaseous changes resulting from the burned candle, which group would benefit the most from a functional candle jar?* (**Hint:** *Think about a gas not involved in aerotolerance.*)

Effect of Environmental Factors on Bacterial Growth

Bacteria have limited control over their internal environments. Whereas many eukaryotes have evolved sophisticated internal control mechanisms, bacteria are almost completely dependent on external factors to provide conditions suitable for their existence. Minor environmental changes can dramatically change a microorganism's ability to transport materials across the membrane, perform complex enzymatic reactions, and maintain critical cytoplasmic pressure.

One way to observe bacterial responses to environmental changes is to artificially manipulate an external factor and measure its effect on growth rate; that is, cell density after a given incubation time.

In this series of laboratory exercises, you will examine the effects of temperature, pH, and osmotic pressure on bacterial growth rate. When appropriate, you will attempt to classify organisms based on your results. ◆

The Effect of Temperature on Microbial Growth

EXERCISE 2-9

Theory

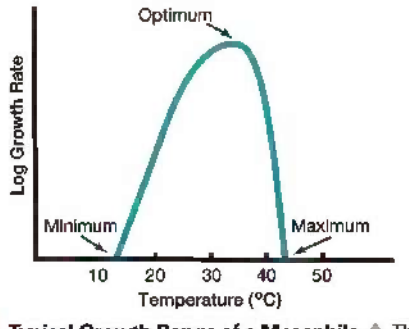
Bacteria have been discovered living in habitats ranging from –10°C to more than 110°C. The temperature range of any single species, however, is a small portion of this overall range. As such, each species is characterized by a minimum, maximum, and optimum temperature—collectively known as its **cardinal temperatures** (Fig. 2.46). Minimum and maximum temperatures are, simply, the temperatures below and above which the organism will not survive. Optimum temperature is the temperature at which an organism grows the fastest—its highest growth rate.

Organisms that grow only below 20°C are called **psychrophiles**. These are common in ocean, Arctic, and Antarctic habitats where the temperature remains permanently cold with little or no fluctuation. Organisms adapted to cold habitats that fluctuate from about 0°C to above 30°C are called **psychrotrophs**. Bacteria adapted to temperatures between 15°C and 45°C are known as **mesophiles**. Most bacterial residents in the human body, as well as numerous human pathogens, are mesophiles.

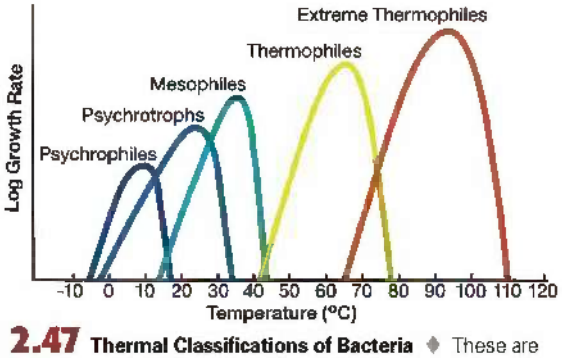
Thermophiles are organisms adapted to temperatures above 40°C. Typically, they are found in composting organic material and in hot springs. Thermophiles that will not grow at temperatures below 40°C are called **obligate thermophiles**; those that will grow below 40°C are known as **facultative thermophiles**. Bacteria isolated from hot ocean floor ridges living between 65°C and 110°C are called **extreme thermophiles**. Extreme thermophiles grow best above 80°C. Figure 2.47 illustrates typical bacterial temperature ranges and classifications.

Application

This is a qualitative procedure designed for observing the effect of temperature on bacterial growth. It allows an



2.46 Typical Growth Range of a Mesophile ◆ The "minimum" and "maximum" are temperatures beyond which no growth takes place. The "optimum" is the temperature at which growth rate is highest.



2.47 Thermal Classifications of Bacteria ◆ These are generic cardinal temperature graphs for each category and represent a composite of all the individual graphs for each species. Refer to the text for a description of each.

estimation of the cardinal temperatures for a single species (rather than for an entire group, as shown in Fig. 2.47).

In This Exercise

Today you will examine the growth characteristics of four organisms at five different temperatures. In addition, you will observe the influence of temperature on pigment production.

✓ Materials

Per Class

- ☐ Five incubating devices set at 10°C, 25°C, 35°C, 45°C, and 60°C. These devices may be any combination of the following:
  - refrigerator
  - incubator
  - hot-water bath
  - cold-water bath

Per Student Group

- ☐ Twenty-one sterile tryptic soy broths (or nutrient broths)
- ☐ Two tryptic soy agar (TSA) plates
- ☐ Four sterile transfer pipettes
- ☐ Fresh trytpic soy broth (or nutrient broth) cultures of:
  - *Escherichia coli*
  - *Geobacillus* (*Bacillus*) *stearothermophilus*
  - *Serratia marcescens*
  - *Pseudomonas fluorescens*

Procedure

Lab One

1. Obtain 21 tryptic soy broths (TSB)—one broth for each organism at each temperature plus one for an uninoculated control. Label them accordingly. Also obtain two TSA plates and label them 20°C and 35°C, respectively.
2. Each student should choose one or two cultures to inoculate from (whatever divides the work load evenly). Mix them thoroughly, but be careful not to splash into the cap.
3. Inoculate five TSB tubes with one drop of organism (be consistent) using a sterile transfer pipette. You may fill the pipette with about 1 mL of broth and deliver the drops with the same pipette to the tubes without flaming them. In your group, leave one tube uninoculated.

   Caution!
   - Insert the pipette well into the tube (but not touching the broth) before delivering the inoculum.
   - Be careful not to spatter as you deliver the drop to each tube, or accidentally drip organism between tubes. Clean up the spill if you do.
   - If you don't have enough organism left in the pipette to inoculate a tube, get more organism. You must not empty ("blow-out") the pipette because you will create aerosols.
   - Properly dispose of the pipette when finished.
4. Using a simple zigzag pattern with a loop, as in Exercise 1-4, inoculate each plate with *Serratia marcescens*.
5. Incubate all tubes in their appropriate temperatures for 24 to 48 hours.
6. Incubate the *Serratia marcescens* plates in the 20°C and 35°C incubators in an inverted position.

Lab Two

1. Clean the outside of all tubes with a tissue, and place them in a test tube rack organized into groups by organism.
2. Mix each broth gently until uniform turbidity is achieved. Be careful not to splash into the caps.
3. Compare all tubes of a species to each other. Rate each as 0, 1, 2, or 3, according to its turbidity (0 is clear—the uninoculated tube—and 3 is highly turbid). Record these in the Broth Data table on the data sheet, page 105.
4. Examine the *Serratia marcescens* plates incubated at two different temperatures, compare the growth characteristics, and enter your results in the Plate Data table on the data sheet.
5. Using the data from the Broth Data table, determine the cardinal temperatures and classification of each of the four organisms.
6. On the graph paper provided with the data sheet, plot the relative turbidity values versus temperature for the four organisms. If you need help with graphing, see pages 9–11.

References

Holt, John G., ed. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Baltimore: Lippincott Williams & Wilkins, 1994.

Moat, Albert G., John W. Foster, and Michael P. Spector. Pages 597–601 in *Microbial Physiology*, 4th ed. New York: Wiley-Liss, 2002.

Prescott, Lansing M., John P. Harley, and Donald A. Klein. Chap. 6 in *Microbiology*, 6th ed. Boston: WCB McGraw-Hill, 2005.

Tille, Patricia M. Page 89 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Varnam, Alan H., and Malcolm G. Evans. *Environmental Microbiology*. Washington, DC: ASM Press, 2000.

White, David. Pages 384–387 in *The Physiology and Biochemistry of Prokaryotes*, 2nd ed. New York: Oxford University Press, 2000.

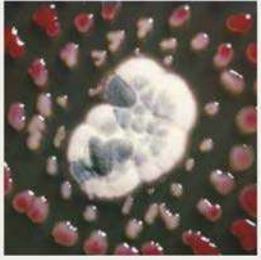
Winn, Washington C. et al. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-9

The Effect of Temperature on Microbial Growth

OBSERVATIONS AND INTERPRETATIONS

1 For each species, record a "3" for the temperature(s) with maximum growth and "0" for no growth. Use "1" and "2" for intermediate amounts of growth.

Broth Data						
Organism	10°C	25°C	35°C	45°C	60°C	Classification

2 Record the growth characteristics of the *Serratia marcescens* incubated at two temperatures.

Plate Data	
Incubation Temperature	Description of Growth
20°C	
35°C	

QUESTIONS

1 *Using the data from the Broth Data table in step 1, determine the cardinal temperatures for each of the four organisms. Circle the optimum temperature for each organism. Use brackets to designate the range for each. Is there any overlap between species? Describe any examples.*

2 *Plot relative turbidity (numeric values) versus temperature on the graph paper provided. See pages 9 through 11 for proper graphing technique.*

3 *Why do different temperatures produce different growth rates?*

4 *Why is it not advisable to connect the data points for each organism in your graph?*

5 *Consider the shape of each curve. Where is the optimum temperature relative to the range for each organism?*

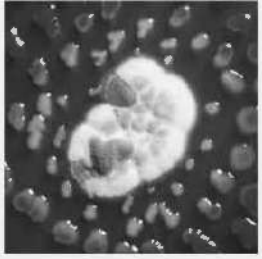
6 *In what way(s) could you adjust the incubation temperature to grow an organism at less than its optimal growth rate?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-9

(continued)

The Effect of pH on Microbial Growth

EXERCISE 2-10

Theory

The conventional means of expressing the concentration (or activity) of hydrogen ions in a solution is "pH." The term *pH*, which stands for *pondus hydrogenii* (variably defined as hydrogen power or hydrogen potential), was invented in 1909 by the Danish biochemist, Søren Peter Lauritz Sørensen. The 0–14 pH range he developed is a logarithmic scale designed to simplify acid and base calculations that otherwise would be expressed as molar values. Sørensen's formula for the calculation of pH is expressed as follows:

$$pH = -\log [H^+]$$

For example, an aqueous solution containing 10^{-6} moles of disassociated hydrogen ions per liter would be converted using Sørensen's formula as follows:

$$pH = -\log [H^+]$$
$$pH = -\log 10^{-6}M\ H^+$$
$$pH = -(-6)$$
$$pH = 6$$

Pure water contains 10^{-7} moles of hydrogen ions per liter and has a pH of 7. As hydrogen ions increase, the solution becomes more acidic and the pH decreases (see Table 2-1).

Bacteria live in habitats throughout the pH spectrum; however, the range of most individual species is small. Like temperature and salinity, pH tolerance is used as a means of classification. The three major classifications are

1. **acidophiles:** organisms adapted to grow well in environments below about pH 5.5,
2. **neutrophiles:** organisms that prefer pH levels between 5.5 and 8.5, and
3. **alkaliphiles:** organisms that live above pH 8.5.

Under normal circumstances, bacteria maintain a near-neutral internal environment regardless of their habitat; pH changes outside an organism's range may destroy necessary membrane potential (in the production of ATP) and damage vital enzymes beyond repair. This **denaturing** of cellular enzymes may be as minor as conformational changes in the proteins' tertiary structure, but usually is lethal to the cell.

Acids from carbohydrate fermentation and alkaline products from protein metabolism are sufficient to disrupt microbial enzyme integrity when grown in vitro. This is why buffers made from weak acids such as hydrogen phosphate are added to bacteriological growth media.

TABLE 2-1 pH Scale

Solution Classification Acidity/Alkalinity	pH	H+ Concentration in Moles/Liter	Common Examples	Organismal Classification
↑ Acidic	0	10^0	Nitric acid	↑
	1	10^{-1}	Stomach acid	
	2	10^{-2}	Lemon juice	
	3	10^{-3}	Vinegar, cola	
	4	10^{-4}	Tomatoes, orange juice	
	5	10^{-5}	Black coffee	Acidophiles
	6	10^{-6}	Urine	
Neutral	7	10^{-7}	Pure water	Neutrophiles
	8	10^{-8}	Seawater	
	9	10^{-9}	Baking soda	Alkaliphiles
	10	10^{-10}	Soap, milk of magnesia	
	11	10^{-11}	Ammonia	
	12	10^{-12}	Lime water [$Ca(OH)_2$]	
	13	10^{-13}	Household bleach	
↓ Alkaline	14	10^{-14}	Drain cleaner	↓

In solution, buffers are able to alternate between weak acid ($H_2PO_4^-$) and conjugate base (HPO_4^{2-}) to maintain H^+/OH^- equilibrium.

$$H^+ + HPO_4^{2-} \longrightarrow H_2PO_4^-$$
$$OH^- + H_2PO_4^- \longrightarrow HPO_4^{2-} + H_2O$$

2

Application

This is a qualitative procedure used to estimate the minimum, maximum, and optimum pH for growth of a bacterial species.

This Exercise

Today you will cultivate and observe the effects of pH on four organisms. Then you will classify them based on your results.

Materials

Per Student Group

- ☐ Five of each pH adjusted tryptic soy broth (or nutrient broth) as follows: pH 2, pH 4, pH 6, pH 8, and pH 10
- ☐ Four sterile transfer pipettes
- ☐ Fresh tryptic soy broth (or nutrient broth) cultures of:
  - *Alcaligenes faecalis* (BSL-2)
  - *Lactobacillus plantarum* or *Lactobacillus acidophilus*
  - *Lactococcus lactis*
  - *Staphylococcus saprophyticus*

Medium Recipe

pH-Adjusted Nutrient Broth

• Beef extract	3.0 g
• Peptone	5.0 g
• Distilled or deionized water	1.0 L
• NaOH or HCl as needed to adjust pH	

Procedure

Lab One

1. Obtain five tubes of each pH broth—one of each pH per organism plus a control (25 tubes total). Label them accordingly.
2. Each student should choose one or two cultures to inoculate from (whatever divides the work load evenly). Mix them thoroughly, but be careful not to splash into the cap.
3. Inoculate one tube of each pH with one drop of the organism (be consistent) using a sterile transfer pipette. You may fill the pipette with about 1 mL of broth and deliver the drops with the same pipette to the tubes without flaming them. **Use BSL-2 precautions when transferring *A. faecalis*, if used.** In your group, leave one tube of each pH uninoculated.

Caution!

- Insert the pipette well into the tube (but not touching the glass or the medium) before delivering the inoculum.
- Be careful not to spatter as you deliver the drop to each tube, or accidentally drip organism between tubes. Clean up the spill if you do.
- If you don't have enough organism left in the pipette to inoculate a tube, get more organism. You must not empty ("blow-out") the pipette because you will create aerosols.
- Properly dispose of the pipette when finished.

4. Incubate all tubes at 35 ± 2°C for 48 hours.

Lab Two

1. Clean the outside of all tubes with a tissue and place them in a test tube rack organized into groups by organism in one direction and pH in the other.
2. Mix each broth gently until uniform turbidity is achieved.
3. Compare all tubes of a species to each other and the appropriate control. Rate each one as 0, 1, 2, or 3 according to its turbidity (0 is clear—the uninoculated tube for each pH—and 3 is highly turbid). Enter your observations in the table on the data sheet, page 111. (*Note:* Some color variability may exist between the different pH broths; therefore, base your conclusions solely on turbidity, not color.)
4. Determine the optimum pH and pH range for each organism. Record the pH category for each on the data sheet.
5. On the graph paper provided with the data sheet, plot the relative turbidity versus pH of the four organisms. If you need help with graphing, see pages 9–11.

References

Forbes, Betty A., Daniel F. Sahm, and Alice S. Weissfeld. Chap. 10 in *Bailey & Scott's Diagnostic Microbiology*, 11th ed. St. Louis, MO: Mosby, 2002.

Holt, John G., ed. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Baltimore: Lippincott Williams & Wilkins, 1994.

Varnam, Alan H. and Malcolm G. Evans. *Environmental Microbiology*. Washington, DC: ASM Press, 2000.

Winn, Washington C. et al. *Koneman's, Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 2-10

The Effect of pH on Microbial Growth

OBSERVATIONS AND INTERPRETATIONS

1 For each species, record a "3" for the pH(s) with maximum growth and "0" for no growth. Use "1" and "2" for intermediate amounts of growth.

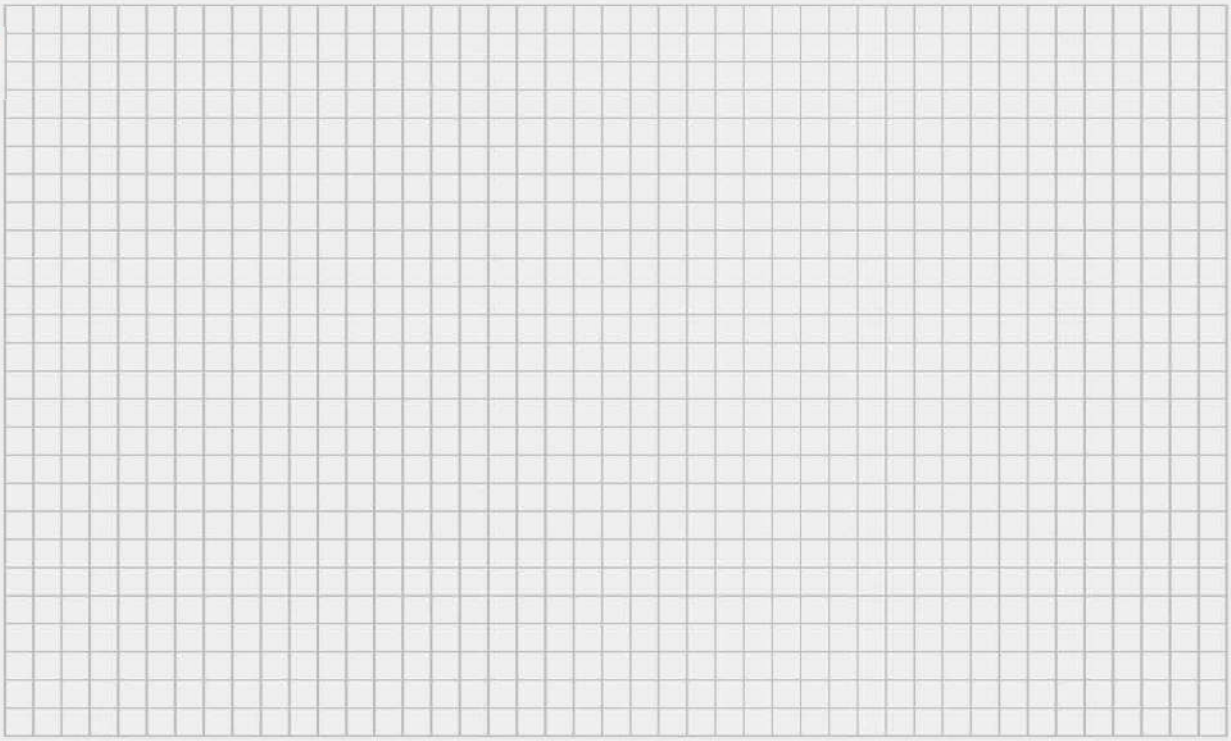
Organism	pH 2	pH 4	pH 6	pH 8	pH 10	Classification

QUESTIONS

1 *Circle the pH optimum for each organism. Place brackets around the range. Is there any overlap between species? Describe any examples.*

2 *Account for the inability of organisms to grow outside their pH ranges. Why, for instance, are alkaliphiles able to survive at high pHs when neutrophiles cannot?*

3 *Plot relative turbidity (numeric values) versus pH for each organism on the graph paper provided. See pages 9 through 11 for proper graphing technique.*



2

4 *Why is it not advisable to connect the data points for each organism in your graph?*

5 *Consider the shape of the four curves.*

a. *Where is the pH optimum relative to the pH range for each organism?*

b. *Do you see any parallels between these data and the data produced in Exercise 2-9? Explain.*

The Effect of Osmotic Pressure on Microbial Growth

EXERCISE 2-11

Theory

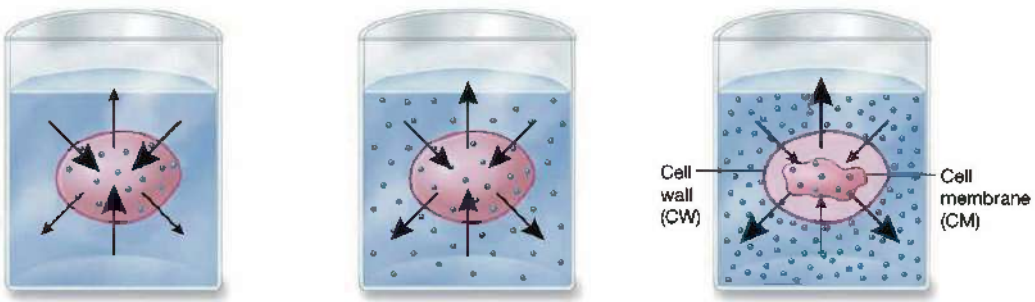
Water is essential to all forms of life. It is not only the principal component of cellular cytoplasm, but also an essential source of electrons and hydrogen ions. Prokaryotes, like plants, require water to maintain cellular **turgor pressure**, that is, internal pressure against the cell wall. Whereas eukaryotic cells burst with a constant influx of water, prokaryotes require water to prevent shrinking of the cell membrane, resulting in separation from the cell wall—an occurrence known as **plasmolysis**.

Many bacteria regulate turgor pressure by transporting in and maintaining a relatively high cytoplasmic potassium or sodium ion concentration, thereby creating a concentration gradient that promotes inward **diffusion** of water. For bacteria living in saline habitats, the job of maintaining turgor pressure is continuous because of the constant efflux of water.

Irrespective of a cell's efforts to control its internal environment, natural forces will cause water to move through its semipermeable membrane from an area of low **solute** concentration to an area of high solute concentration. In a solution in which solute concentration is low, water concentration is high, and vice versa. Therefore, water moves through a cell membrane from where its concentration is high to where its concentration is low. This process is called **osmosis**, and the force that controls it is called **osmotic pressure**.

Osmotic pressure is a quantifiable term and refers, specifically, to the ability of a solution to *pull water toward itself* through a semipermeable membrane. If a bacterial cell is placed into a solution that is **hyposmotic** (a solution having low osmotic pressure), there will be a *net* movement of water into the cell—at least until internal hydrostatic pressure equals osmotic pressure. At this point, net water movement stops because a dynamic equilibrium is reached. If an organism is placed into a **hyperosmotic** solution (a solution having high osmotic pressure), there will be a net movement of water out of the cell. For a bacterial cell in an **isosmotic** solution (a solution having osmotic pressure equal to that of the cell), water will tend to move in both directions equally; that is, there is no net movement (Fig. 2.48).

Bacteria constitute a diverse group of organisms and, as such, have evolved many adaptations for survival. Microorganisms tend to have a distinct range of salinities that are optimal for growth, with little or no survival outside that range. For example, some bacteria called **halophiles** grow optimally in NaCl concentrations of 3% or higher. **Extreme halophiles** are organisms with specialized cell membranes and enzymes that require salt concentrations from 15% up to about 25% and will not survive where salinity is lower (Fig. 2.49). Except for a few **osmotolerant** bacteria, which will grow over a wide range of salinities, most bacteria live where NaCl concentrations are less than 3%.



2.48 The Effect of Osmotic Pressure on Bacterial Cells ♦ This osmosis diagram illustrates the movement of water into and out of cells. The labels refer to the osmotic pressure of the solution outside the cell. In a **hyposmotic** environment, the cell has greater osmotic pressure, so the net movement of water (arrows) will be into the cell. In an **isosmotic** environment, there is no net movement because the osmotic pressure of the cell and that of the environment are equal. (Actually, water molecules are moving equally in both directions.) In a **hyperosmotic** environment, the osmotic pressure of the environment is greater, so the net water movement is outward and results in plasmolysis. Note the shrinking membrane (CM) and the rigid cell wall (CW) in the hyperosmotic solution.

2.49 A Saltern in San Diego Bay ♦ Salterns are low pools of saltwater used in the harvesting of salt. As water evaporates, the saltwater becomes saltier and saltier, until only salt remains. This can then be purified and sold. The colors in the pools result from different communities of halophilic microorganisms that are associated with different salinities as the pools dry out.

Application

This is a qualitative procedure used to demonstrate bacterial tolerances to NaCl.

In This Exercise

You will be growing three microorganisms at a variety of NaCl concentrations to determine the salinity tolerance range and optimum salinity for each organism.

✓ Materials

Per Student Group

- ☐ Four tubes each of saline medium prepared with 0%, 5%, 10%, 15%, 20%, and 25% NaCl
- ☐ Three sterile transfer pipettes
- ☐ Fresh tryptic soy broth (or nutrient broth) cultures of:
  - *Escherichia coli*
  - *Halobacterium salinarum*
  - *Staphylococcus epidermidis*

Medium Recipe

Modified *Halobacterium* Broth

• Sodium chloride	0 g, 50 g, 100 g, 150 g, 200 g, or 250 g
• Magnesium sulfate, heptahydrate	20.0 g
• Trisodium citrate, dihydrate	3.0 g
• Potassium chloride	2.0 g
• Casamino acids	5.0 g
• Yeast extract	5.0 g
• Deionized water	1.0 L

Adjust pH to 7.2 using 5 M or concentrated HCl

Procedure

Lab One

1. Obtain four tubes of each *Halobacterium* broth—one of each concentration per organism plus a control (24 tubes total). Label them accordingly.
2. Each student should choose one or two cultures to inoculate from (whatever divides the work load evenly). Mix them thoroughly, but be careful not to splash into the cap.
3. Inoculate one tube of each salinity with one drop of the organism (be consistent) using a sterile transfer pipette. You may fill the pipette with about 1 mL of broth and deliver the drops with the same pipette to the tubes without flaming them. In your group, leave one tube of each salinity uninoculated.

   Caution!

   - Insert the pipette well into the tube (but not touching the glass or the medium) before delivering the inoculum.
   - Be careful not to spatter as you deliver the drop to each tube, or accidentally drip organism between tubes. Clean up the spill if you do.
   - If you don't have enough organism left in the pipette to inoculate a tube, get more organism. You must not empty ("blow-out") the pipette because you will create aerosols.
   - Properly dispose of the pipette when finished.
4. Incubate all tubes at 35 ± 2°C for 48 hours (or longer, if necessary).

Lab Two

1. Clean the outside of all tubes with a tissue, and place them in a test tube rack organized into groups by organism in one direction and by salinity in the other.
2. Mix each broth gently until uniform turbidity is achieved.
3. Compare all tubes of a species to each other and to the control. Rate each one as 0, 1, 2, or 3 according to its turbidity (0 is clear—the uninoculated tube for each salinity—and 3 is highly turbid). Enter your observations in the table on the data sheet, page 117. (*Note:* The different broths may show some color variability. Check the uninoculated controls and, thus, base your conclusions solely on turbidity, not color.)
4. From your data, determine the optimum salinity and salinity range for each organism.
5. On the graph paper provided with the data sheet, plot the relative turbidity versus the salt concentration for all three organisms. If you need help with graphing, see pages 9 through 11.

References

Forbes, Betty A., Daniel F. Sahm, and Alice S. Weissfeld. Chap. 10 in *Bailey & Scott's Diagnostic Microbiology,* 11th ed. St. Louis, MO: Mosby, 2002.

Hauser, Juliana T. *Techniques for Studying Bacteria and Fungi.* Burlington, NC: Carolina Biological Supply Company, 2006.

Holt, John G., Ed. *Bergey's Manual of Determinative Bacteriology,* 9th ed. Baltimore: Lippincott Williams & Wilkins, 1994.

Koneman, Elmer W., Stephen D. Allen, William M. Janda, Paul C. Schreckenberger, and Washington C. Winn, Jr. *Color Atlas and Textbook of Diagnostic Microbiology,* 5th ed. Philadelphia: J. B. Lippincott Co., 1997.

Moat, Albert G., John W. Foster, and Michael P. Spector. Pages 582–587 in *Microbial Physiology,* 4th ed. New York: Wiley-Liss, 2002.

Varnam, Alan H. and Malcolm G. Evans. *Environmental Microbiology.* Washington, DC: ASM Press, 2000.

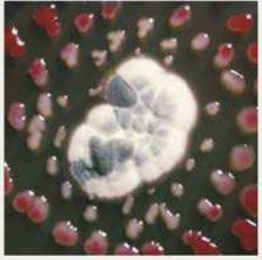
White, David. Pages 388–394 in *The Physiology and Biochemistry of Prokaryotes,* 2nd ed. New York: Oxford University Press, 2000.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-11

The Effect of Osmotic Pressure on Microbial Growth

OBSERVATIONS AND INTERPRETATIONS

1 For each species, record a "3" for the salt concentration(s) with maximum growth and "0" for no growth. Use "1" and "2" for intermediate amounts of growth.

	NaCl Concentration					
Organism	**0%**	**5%**	**10%**	**15%**	**20%**	**25%**
Control						

QUESTIONS

1 *Circle the optimum salinity for each organism. Place brackets around the range. Is there any overlap between species? Describe any examples.*

2 *Plot relative turbidity (numeric values) versus salt concentration on the graph paper provided. See pages 9 through 11 for proper graphing techniques.*

3 *Why is it not advisable to connect the data points for each organism in your graph?*

2

4 *Which organism(s) exhibit(s) the greatest tolerance range?*

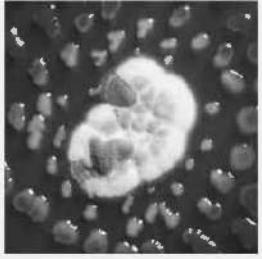
5 *Use your textbook or other available references to look up the habitat of each organism. Do the habitats make sense in light of the tolerance ranges you obtained? Explain your answers.*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-11

(continued)

Control of Pathogens: Physical and Chemical Methods

Every patient in a hospital or other clinical setting has the right to expect that he or she will not contract a disease or infection while in that institution's care. Every person donating blood at a blood bank or mobile center has the right to expect that all materials and surfaces they come in contact with will be free of pathogens. Workers in health clinics, hospitals, medical laboratories, and public health laboratories have the right to assume that reasonable precautions have been and are being taken to protect their safety while in the workplace.

These are only a few of the many reasons why the importance of understanding and use of pathogen control systems cannot be overemphasized. Fortunately, with relatively few exceptions, the above-described conditions exist in this and other developed countries largely because of the dedication of thousands of employees and the oversight of dozens of international, governmental, and private organizations such as the World Health Organization (WHO), Centers For Disease Control and Prevention (CDC), Food and Drug Administration (FDA), Environmental Protection Agency (EPA), American Public Health Association (APHA), and Association of Official Analytical Chemists (AOAC). These and many other federal and private organizations are responsible for the proper testing, registration, and classification of the substances or systems used to prevent the spread of pathogens.

These substances or systems, both chemical and physical, are referred to broadly as germicides. Some germicides are specific in nature and typically include the name of the target pathogen, such as "tuberculocide," "virucide," or "sporocide." Most germicides are broad-spectrum and, thus, target a wide variety of pathogens. Although some overlap occurs, germicidal systems fall into three categories: decontamination, disinfection, or sterilization.

1. **Decontamination** is the lowest level of control and is defined as "reduction of pathogenic microorganisms to a level at which items are safe to handle without protective attire." Decontamination usually includes physical cleaning with soaps or detergents, and removal of all (ideally) or most organic and inorganic material. Proper cleaning of all instruments and surfaces is considered the critical first step toward disinfection or sterilization because, to be fully effective, a disinfectant or sterilant must come in direct contact with all pathogens present. Materials left to dry on a surface or apparatus can actually shield pathogens from a disinfecting or sterilizing agent or otherwise neutralize it.

2. **Disinfection** is the next level of control and is divided into three sublevels—low, medium, and high—based on effectiveness against specific control pathogens or their surrogates. All sublevels kill large numbers, if not all, of the targeted pathogens but typically do not kill large numbers of bacterial endospores. Some high-level disinfectants are called chemical sterilants because they have the ability to kill all vegetative cells and endospores.

   Disinfectants typically are liquid chemical agents but can also be solid or gaseous. Other disinfection methods include dry heat, moist heat, and ultraviolet light. Disinfectants that are designed to reduce or eliminate pathogens on or in living tissue are called **antiseptics**. For obvious safety reasons, antiseptics are subject to additional testing to minimize the risks of side effects. Some antiseptics are considered drugs and, therefore, are regulated by the FDA.

3. **Sterilization** is the complete elimination of viable organisms including bacterial endospores and, as such, is the highest level of pathogen control. Sterilization can be achieved by some chemicals, some gases, incineration, dry heat, moist heat, ethylene oxide gas, ionizing radiation (gamma, X-ray, and electron beam), low-temperature plasma (utilizing a combination of chemical sterilants and ultraviolet radiation in a vacuum chamber), or low-temperature ozone (utilizing bottled oxygen, water, and electricity in a chamber to produce a lethal level of ozone).

In this last portion of Section 2 you will examine one final aspect of microbial growth: its control, by both physical and chemical means. The following exercises illustrate the germicidal effects of UV radiation, disinfection, antisepsis, and steam (moist heat) sterilization. (For more information on microbial control, refer to Exercise 7-3 Antimicrobial Susceptibility Test.) ◆

Steam Sterilization

Theory

Of the many methods or agents that have been developed for sterilizing surgical and dental instruments, microbiological media, infectious waste, and other materials not harmed by moisture or heat, steam is still the most effective and most common. The device used most commonly for this purpose is called a steam sterilizer, or **autoclave**. Autoclaves are relatively safe, easy to operate, and, if used properly, effective at killing all microbial vegetative cells and bacterial endospores.[1]

Under atmospheric pressure, water boils at 100°C (212°F). At pressures above atmospheric pressure, water must be heated above 100°C before it will boil. Similar to home pressure cookers, which create pressure and high temperatures to shorten cooking times, autoclaves use super-heated steam under pressure to kill heat-resistant organisms. Examples of heat-resistant organisms include members of the spore-producing genera—*Bacillus*, *Geobacillus*, and *Clostridium*.

In the microbiology laboratory, sterilizing temperature usually is set at between 121°C and 127°C (250°F and 260°F); however, sterilizing time can vary according to the size and consistency of the material being sterilized.[2] At a minimum, to be sure that all vegetative cells and endospores have been killed, items being processed must reach optimum temperature for at least 15 minutes. This includes items deep inside the autoclave container that may be partially insulated from the steam by surrounding items. Understandably, larger loads take longer to process than smaller loads. (Certain sensitive applications, such as microbiological media preparation, in which formula integrity must be maintained and when specific growth inhibiting ingredients are included, lower times and temperatures are acceptable.)

To maintain laboratory safety and comply with laws regarding infectious waste disposal, sterilizers must be checked regularly for operating effectiveness. Special thermometers placed in an autoclave can record the maximum temperature reached inside the chamber but do not measure how low the temperature dips during

2.50 Autoclave Tape ♦ A commonly used method of verifying that sterile conditions have been achieved in an autoclave is to place a strip of autoclave tape on the item(s) to be sterilized. Prior to autoclaving, autoclave tape looks very much like masking tape. However, if 121°C for 15 minutes has been produced inside the autoclave, black stripes appear on the tape.

2.51 Autoclave Biological Indicators ♦ These indicator vials contain an ampule of fermentation broth and a filter paper strip containing endospores of *Geobacillus stearothermophilus*—a spore-forming organism capable of withstanding high temperatures. The vial in the center was autoclaved for 15 minutes at 121°C, cooled, pinched to crush the inner glass ampule, and incubated. The purple color (compared to the uncrushed negative control on the right) indicates that an acidic condition from fermentation does not exist. This suggests that the organism has been killed by the autoclaving. Note the gray-colored band on the label. This chemical indictor changes from blue to gray upon autoclaving. The ampule in the negative control was not crushed, so the spores in the filter paper never made contact with the broth and, thus, provides a color example of unfermented broth. The ampule on the left is a positive control to verify the viability of the organism used in the system. It was not autoclaved (as evidenced by the blue band on the label) but was crushed and incubated. The development of yellow color indicates that the organism in the system is viable and that the lack of yellow color in the center vial is a result of autoclaving.

[1] Steam sterilization is not yet considered reliable at inactivating prion proteins, such as those that cause bovine spongiform encephalopathy, the so-called mad cow disease, and its variant, Creutzfeldt-Jakob disease.

[2] In clinics, hospitals, or other locations where surgical instruments are being processed, the World Health Organization (WHO) recommends a minimum processing time and temperature of 134°C for 18 minutes.

the normal cycling of the heating elements. Specialized color-coded autoclave tape (Fig. 2.50) can be a fairly good indicator that sterilization is complete, but the only way, with certainty, to determine that sterilization has been achieved is by using a device called a **biological indicator** (Fig. 2.51).

Biological indicators, as the name suggests, are test systems that contain something living. A typical biological indicator that is particularly useful for testing autoclaves is one that contains **bacterial endospores**. Bacterial endospores, the dormant form of an organism, are highly resistant to both chemical and physical means of control. Therefore, if an autoclave kills the endospores in the test system, it is safe to assume that it will destroy other microbes as well. This is important not only for safety reasons but is of legal importance as well. Public health and safety agencies maintain compliance with hazardous waste disposal regulations by requiring regular testing of autoclaves used to process biohazardous material.

A typical system, and the one selected for today's lab, includes a small, heat-resistant plastic vial containing a glass ampule of sterile fermentation broth. Also inside the vial, but outside of the ampule, is a strip of filter paper containing bacterial endospores. The vial is placed inside the autoclave and heated at 121°C for 15 minutes. After autoclaving, the vial is cooled and crushed with a special device that breaks the inner ampule without damaging the plastic vial. Breaking the ampule allows the fermentation broth to come in contact with the bacterial endospores in the filter paper. The vials are then incubated at 55°C for 48 hours. If the endospores have been killed in the autoclave, incubation will produce no growth. If they have not been killed, they will germinate and ferment the substrate in the broth. A pH-indicating dye, included in the broth, will reveal any acid produced (during fermentation) with a distinctive color change. No color change during incubation is, thus, an indication that sterilization is complete, the endospores have been killed, and the autoclave is operating properly. (For more information on fermentation, refer to Section 5 and Appendix A.)

Application

Biological indicators are available in many forms and commonly used to test the efficiency of steam sterilizers.

In This Exercise

You are going to use bacterial endospores and their resistance to steam sterilization to test the effectiveness of your lab's autoclave. This procedure is written for a product called BTSure Biological Indicator[3], but can be applied to other brands as well. BTSure Biological Indicators include a pH indicator that turns the broth from purple to yellow under the acidic conditions produced by fermentation.

[3] BTSure Biological Indicators are available from Fisher Science, http://www.fishersci.com/ecomm/servlet/fsproductdetail_10652_684255__-1_0.

✓ Materials

Per Class

- ☐ One (or more) steam autoclave(s)
- ☐ Incubator set at 55°C

Per Student Group

- ☐ Four BTSure Biological Indicators
- ☐ One autoclave pan

Procedure

1. Obtain four BTSure Biological Indicator vials and label them #1, #2, #3, and #4 with a Sharpie® or other permanent marker. Do not label the vial with tape or paper, because this will insulate it from the steam.
2. Place vial #1 on its side uncovered in an autoclave pan.
3. Place vial #2 inside a container (or multiple containers), as well insulated as can be achieved with materials provided by your instructor. We recommend placing the vial inside a screw-capped test tube inside other tubes—two, three, or four layers deep. Place this vial in the autoclave pan with vial #1.
4. Do nothing with vials #3 and #4 as yet.
5. Place the autoclave pan in the autoclave.

6 Follow your instructor's guidelines to add water to the chamber, set the temperature at 121°C (250°F), and set the timer at 15 minutes. When instructed to do so, close and start the autoclave.

7 When autoclaving is complete, all of the steam has been vented, and the machine has been allowed to cool slightly, remove your pan (while protecting your hands with appropriate gloves) and allow the contents to cool to room temperature.

8 Keeping the vials in an upright position, use the crushing device to squeeze vials #1, #2, and #3 (one at a time) until you hear the glass ampule inside the vial break.

9 Place these vials along with vial #4, again in an upright position, into the 55°C incubator for 48 hours.

10 After incubation, examine the vials for color changes.

11 Using Table 2-2 as a guide, record and interpret your results in the table provided on the data sheet, page 127.

TABLE 2-2 **Autoclave Biological Indicator Test Results and Interpretations**

Broth Color	Interpretation
Purple	No fermentation or acid production in the medium. The organism is dead.
Yellow	Fermentation with acid production in the medium. The organism is alive.

(Assume the ampules inside the vials have been crushed)

References

McDonnell, Gerald E. *Antisepsis, Disinfection, and Sterilization: Types, Action, and Resistance.* Washington, DC: ASM Press, American Society for Microbiology, 2007.

Widmer, Andreas F. and Reno Frei. Chap. 7 in *Manual of Clinical Microbiology,* 9th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Marie Louise Landry, and Michael A. Pfaller, eds. Washington, DC: ASM Press, American Society for Microbiology, 2007.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 2-12

Steam Sterilization

OBSERVATIONS AND INTERPRETATIONS

1 Examine all vials and record your results in the table below.

Indicator Vial	Color Result	Interpretation
Vial #1		
Vial #2		
Vial #3		
Vial #4		

QUESTIONS

1 *Why are the bacterial endospores placed on the paper strip and not directly in the fermentation broth?*

2 *What is the purpose of the unautoclaved/unbroken vial?*

3 *How would you interpret the following combinations of results? Vial numbers indicate the treatment as in this experiment.*

a. *Vial #1—purple, vial #2—purple, vial #3—purple, vial #4—purple?*

b. *Vial #1—purple, vial #2—purple, vial #3—yellow, vial #4—purple?*

c. *Vial #1—purple, vial #2—yellow, vial #3—yellow, vial #4—purple?*

d. *Vial #1—yellow, vial #2—yellow, vial #3—yellow, vial #4—purple?*

e. *Vial #1—yellow, vial #2—yellow, vial #3—yellow, vial #4—yellow?*

4 *What changes would you make to avoid repeating the faulty scenarios illustrated in question 3?*

The Lethal Effect of Ultraviolet Radiation on Microbial Growth

EXERCISE 2-13

Theory

Ultraviolet radiation (UV light) is a type of **electromagnetic energy**. Like all electromagnetic energy, UV travels in waves and is distinguishable from all others by its **wavelength**. Wavelength is the distance between adjacent wave crests and is typically measured in nanometers (nm) (Fig. 2.52).

Ultraviolet light is divided into three groups categorized by wavelength:

1. UV-A, the longest wavelengths, ranging from 315 nm to 400 nm
2. UV-B, wavelengths between 280 nm and 315 nm
3. UV-C, wavelengths ranging from 100 nm to 280 nm. (These wavelengths—more specifically, 240 nm–280 nm—are most detrimental to bacteria. Bacterial exposure to UV-C for more than a few minutes usually results in irreparable DNA damage and death of the organism. For a discussion on the mutagenic effects of UV and DNA repair, refer to Exercise 10-5.)

The germicidal effect of UV-C is related to time of exposure, lamp intensity, and distance to the target (the inverse square law applies here—intensity diminishes by the reciprocal of square of the distance: $\frac{1}{\text{distance}^2}$). It also must have "line of sight" to the surface being decontaminated. That is, it doesn't penetrate well, bend around corners, or trickle into crevices. Lastly, its effectiveness is diminished by dust, organic material, etc. Taken together, UV-C as a germicidal agent has its limited applications.

Application

Ultraviolet light is commonly used to disinfect laboratory and health-care environment work surfaces and surrounding air.

In This Exercise

Today you will compare the effect of UV exposure on two (or three) cultures—*Bacillus subtilis* and *Escherichia coli*. Because of the large number of plates to be treated, the work will be divided among six groups of students. Refer to Table 2-3 for assignments.

✓ Materials

Per Student Group

- ☐ Short wavelength ultraviolet lamp (UV-C) with appropriate shielding and support
- ☐ Cardboard to cover plates
- ☐ Two tryptic soy agar (TSA) plates (four for group one)
- ☐ Stopwatch or electronic timer
- ☐ Sterile cotton swabs (two per group; four for group 1)
- ☐ 24-hour tryptic soy broth cultures of:
  - *Bacillus subtilis*
  - *Escherichia coli*
- ☐ (Optional) 7-day *Bacillus subtilis* TSB culture

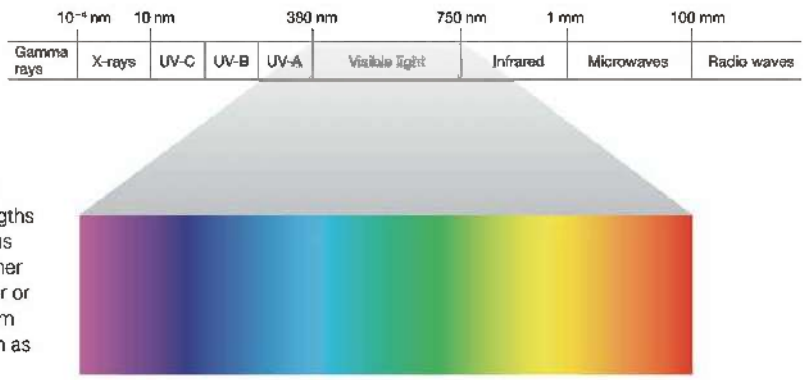
Procedure

Lab One

1. Enter your group number and exposure time (from Table 2-3) on the data sheet, page 131.
2. Obtain two TSA plates and label the bottom of each with the name of the organism to be inoculated and your group number. Draw a line to divide the plates in half, and label the sides "A" and "B."

TABLE 2-3 **Group Assignments by Number**

Organism	No UV	1 minute	2 minutes	4 minutes	8 minutes	12 minutes	16 minutes
B. subtilis (24-hour culture)	1	1	2	3	4	5	6
E. coli	1	1	2	3	4	5	6
B. subtilis (7-day culture)	1	1	2	3	4	5	6



2.52 Electromagnetic Spectrum ♦ The shortest and highest energy wavelengths are those of gamma rays with wavelengths less than 10^{-4} nm. Radio waves, at the other end of the spectrum, can be one kilometer or longer. Between about 100 nm and 380 nm (just above visible light) is the sliver known as ultraviolet light.

3. Dip a sterile cotton swab into the broth of one culture and wipe the excess on the inside of the tube. Inoculate the appropriate plate by spreading the organism over the entire surface of the agar. Do this by streaking the plate surface completely three times, rotating it one-third turn between streaks. When incubated, this will form a bacterial lawn.
4. Repeat step 3 with the other organism(s) and plate.
5. Place a paper towel on the table next to the UV lamp and soak it with disinfectant.
6. Place your plates under the UV lamp and set the covers, open side down, on the disinfectant-soaked towel. Cover the B half of the plates with the cardboard as shown in Figure 2.53.
7. Be sure the lamp is the same distance from the plates for all groups (we recommend between 8 and 12 inches). Then, turn on the lamp for the prescribed time. ***Caution:*** **Be sure the protective shield is in place and do not look at the light while it is on!**
8. Immediately replace the covers, invert the plates and incubate them at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Remove your plates from the incubator and observe for growth. Side B should be covered with a bacterial lawn. If this is not the case, see your instructor.
2. Record the growth on side A of each plate in the table on the data sheet, page 131. Enter "0" if you observe no growth, "1" for poor growth, "2" for moderate growth, and "3" for abundant growth.
3. Using the results from other groups, complete the class data table on the data sheet.
4. On the graph paper provided with the data sheet, construct a graph representing growth versus UV exposure time for all three organisms. If you need help with graphing, see pages 9 through 11.

2.53 Plates Shielded for UV Exposure ♦ Place the two plates under the UV lamp with the covers removed and the cardboard shield covering half of each plate as shown. Make sure the Petri dish covers are placed open side down on a disinfectant-soaked towel.

References

"Guideline for Disinfection and Sterilization in Healthcare Facilities, 2008," Centers for Disease Control and Prevention, Healthcare Infection Control Practices Advisory Committee (HICPAC), http://www.cdc.gov/hicpac/Disinfection_Sterilization/10_0MiscAgents.html#2.

Krebs, Jocelyn E., Elliott S. Goldstein, and Stephen T. Kilpatrick. Chap. 16 in *Lewin's Genes XI*. Burlington, MA: Jones & Bartlett Learning, 2012.

Varnam, Alan H., and Malcolm G. Evans. *Environmental Microbiology*. Washington, DC: ASM Press, 2000.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 2-13

The Lethal Effect of Ultraviolet Light on Microbial Growth

OBSERVATIONS AND INTERPRETATIONS

1 Enter your class data in the table below. Score the relative amount of growth on each plate using the following scale:

0 = no growth

1 = individual colonies

2 = sparse confluent growth

3 = heavy confluent growth

UV Exposure (minutes)	*E. coli*		*B. subtilis* (24-hour culture)		*B. subtilis* (7-day culture)	
	Side A	Side B	Side A	Side B	Side A	Side B

QUESTIONS

1 *The purpose of this exercise is to demonstrate the comparative effect of UV on two (or three) bacterial populations. This could have been accomplished without the cardboard cover. Why was the cover used?*

2 *This is not a quantitative exercise. Keeping this in mind, can you see a general trend between bacterial death and UV exposure time?*

3 *Which organism survived the longest exposure? Why?*

4 *Why were you told to remove the plate covers prior to exposing them to UV?*

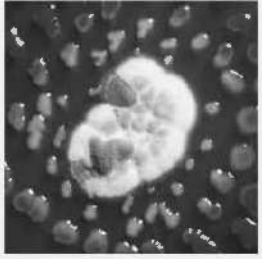
5 *Using the graph paper provided, construct a single graph of relative growth (0, 1, 2, 3) versus UV exposure time for the two organisms. See pages 9 through 11 for proper graphing technique.*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 2-13

(continued)

Chemical Germicides: Disinfectants and Antiseptics

EXERCISE 2-14

Theory

Chemical germicides are substances designed to reduce the number of pathogens on a surface, in a liquid, or on or in living tissue. Germicides designed for use on surfaces (floors, tables, sinks, countertops, surgical instruments, etc.) or in liquids are called **disinfectants**. Germicides designed for use on or in living tissue are called **antiseptics**.

Before a new substance can be registered by either the FDA or EPA and allowed on the market, it must be tested and classified according to its effectiveness against pathogens. The Use-Dilution Test, published by the Association of Official Analytical Chemists (AOAC), is one of many commonly used tests for this purpose.

The Use-Dilution Test is a standard procedure used to measure the effectiveness of disinfectants specifically against *Staphylococcus aureus*, *Salmonella enterica* serovar Cholerasuis, and *Pseudomonas aeruginosa*. In the standard procedure, glass beads or stainless steel cylinders coated with living bacteria are exposed to varying concentrations (dilutions) of test germicides then transferred to a growth medium. After a period of incubation, the medium is examined for growth. If a solution is sufficient to prevent microbial growth at least 95% of the time, it meets the required standards and is considered a usable dilution of that germicide for a specific application. Today's exercise is an adaptation of this method.

Application

This procedure is used to test the effectiveness of germicides against *Salmonella* spp., *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

In This Exercise

Today you will examine the effectiveness of four germicides—two common household disinfectants and two over-the-counter antiseptics. The disinfectants selected for the exercise are household bleach and Lysol® Brand II Disinfectant. The antiseptics are hydrogen peroxide and isopropyl alcohol. The organisms used for the test are *Staphylococcus epidermidis* and *Escherichia coli*. (The usual test organisms are BSL-2 and we can demonstrate the protocol without using them.)

You will first coat the beads with bacteria, expose them to three concentrations of your assigned germicide, and then use them to inoculate sterile nutrient broth. If, during exposure to the germicide, all of the bacteria on the bead are killed the broth inoculated with that bead will remain clear. If any of the bacteria survive the germicide exposure, they will reproduce during incubation and make the broth turbid. With these results, you will determine the effective concentration (dilution) of your germicide.

The tasks for the exercise are divided among eight groups of students (or a convenient number for your lab size). Each group will be responsible for one organism and three dilutions of one germicide. Refer to Table 2-4 for your assignments.

Finally, this is an interesting exercise with moderate amount of work involved. If you do not hurry and are careful to use aseptic technique, you will be rewarded with reliable data at the end.

TABLE 2-4 Group Assignments

Germicide	*Staphylococcus epidermidis*	*Escherichia coli*
Bleach	Group 1	Group 2
Lysol®	Group 3	Group 4
Hydrogen peroxide	Group 5	Group 6
Isopropyl alcohol	Group 7	Group 8

✓ Materials

Per Class (see Table 2-4)

Disinfectants

- ☐ 0.01%, 0.1%, and 1% household bleach
- ☐ 25%, 50%, and 100% Lysol® Brand II Disinfectant

Antiseptics

- ☐ 0.03%, 0.3%, and 3% hydrogen peroxide (3% is full strength as purchased at the pharmacy)
- ☐ 10%, 30%, and 50% isopropyl alcohol (70% is full strength as purchased at the pharmacy)

Per Student Group

- ☐ 100 mL flask of sterile deionized water
- ☐ Three concentrations of one germicide (listed above)
- ☐ Five sterile 60 mm Petri dishes
- ☐ One sterile glass 100 mm Petri dish containing filter or bibulous paper

- ☐ One container of sterile ceramic or glass beads[1]
- ☐ Sterile transfer pipette
- ☐ Seven sterile nutrient broth tubes
- ☐ Needle-nose forceps (or appropriate device for aseptically picking up beads)
- ☐ Small beaker with alcohol (for flaming forceps)
- ☐ Fresh broth cultures of (one per group):
  - *Escherichia coli*
  - *Staphylococcus epidermidis*

Procedure[2]

Timing is important in this procedure. Read through it and make a plan before you begin so your transfers and soaking times are done uniformly and are consistent with those of other groups.

Lab One

1. Enter the name of your organism here:

   _____.

2. Enter the name of your germicide here:

   _____.

3. Obtain all of the necessary items for your group as listed in Materials.
4. Place the materials properly on your workspace as shown in Figure 2.54. Label all seven broths with your group name or number. Label three of the broths "Broths #1, #2, and #3" and label the last four broths "Controls #1, #2, #3, and #4" as shown in the diagram in Figure 2.54.
5. Label three of the 60 mm plates with the name and concentration of your germicide. Label the other two plates "#4 sterile water" and "#5 sterile water."
6. Add approximately 15 mL of each germicide concentration to its respective plate (Plates #1 through #3). Add approximately 15 mL sterile water into Plates #4 and #5.
7. Carefully mix your bacterial culture until uniform turbidity is achieved. Take care not to splash into the cap.
8. Aseptically transfer one loopful of culture broth to Control #2.
9. Alcohol-flame your forceps and aseptically drop six beads into the broth culture. (You will only use four beads; the other two are extras in case you drop one during the procedure.) *Note:* Store your forceps in the beaker of alcohol between transfers. When it is time to make a transfer, remove the forceps and burn off the alcohol, holding them away from the alcohol beaker while doing so. If the alcohol beaker catches on fire, smother the flame with the lid. When finished, return the forceps directly to the beaker without flaming.
10. After 1 minute, decant the broth into a beaker of disinfectant. Remove as much of the broth as possible without losing the beads in the disinfectant.
11. Dispense the beads onto the sterile filter paper. This can be done by tapping the mouth of the tube on the paper. If this doesn't work, remove the beads with a sterile inoculating loop and flame it afterward
12. Using alcohol-flamed forceps spread the beads apart on the paper and allow them to dry. Do not roll them around because this may remove bacteria.
13. After 10 minutes, place one bead in each of the three germicide plates using alcohol-flamed forceps. Mark the time here:

    _____.

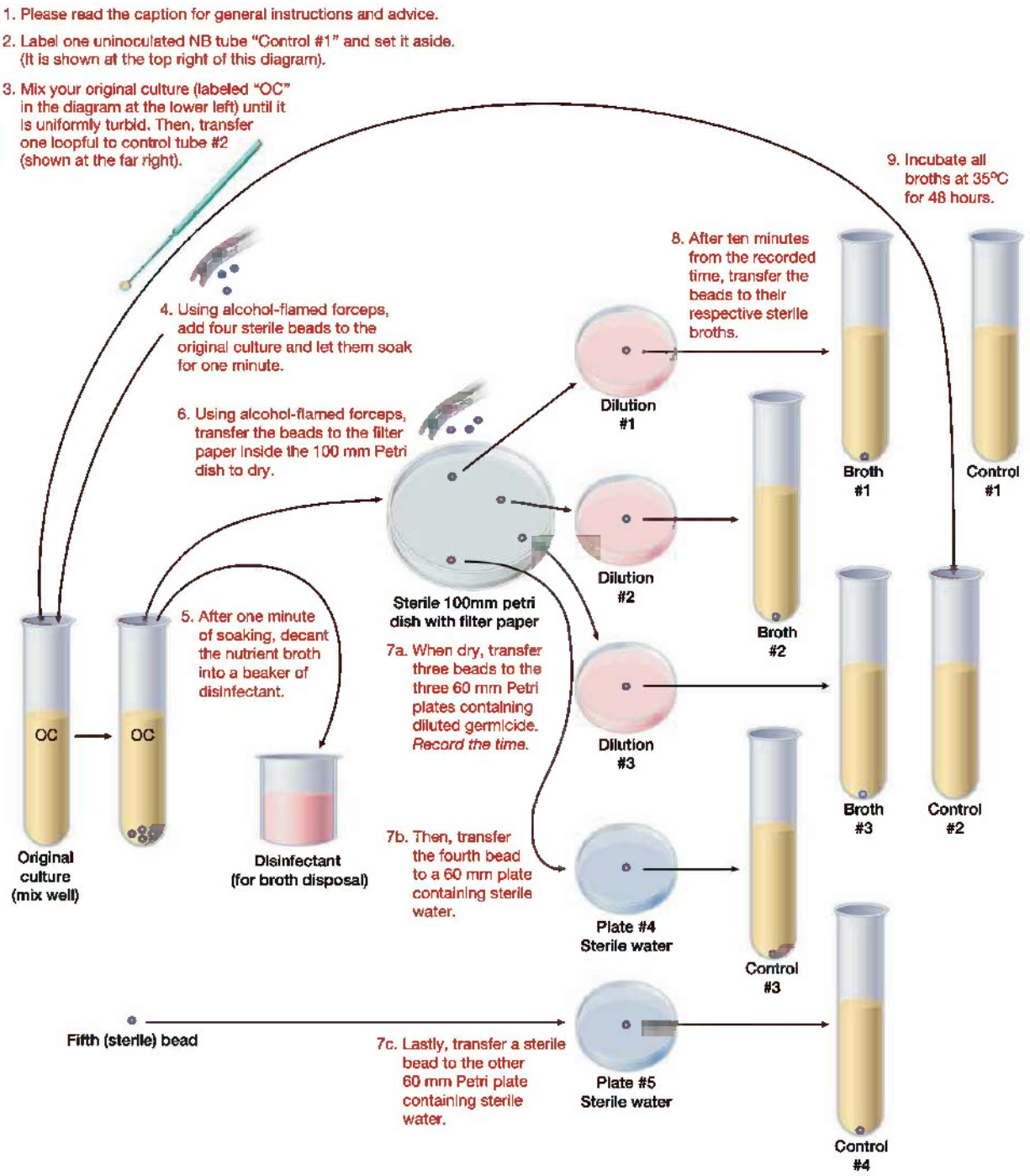
14. With alcohol-flamed forceps, place the fourth bead in plate #4 (sterile water).
15. With alcohol-flamed forceps, place a sterile bead in plate #5 (sterile water).
16. After 10 minutes from the time marked in step 13, remove the beads from the solutions, in the same order as they were added, and place them in their respective nutrient broths. Carefully mix the broths immediately to disperse any residual disinfectant on the beads. Do not splash the broth into the cap.
17. Incubate all seven broths at 35 ± 2°C for 48 hours.

[1] Sterilized #8 seed beads from a craft store will work for this purpose.

[2] This procedure has been modified from its original form and is to be used for instructional purposes only.

Lab Two

1. Remove all broth tubes from the incubator. Gently mix the controls and examine them for evidence of growth. Enter your results on the data sheet, page 139. Control #1 should have produced growth (turbidity) and Control #2 should not have produced growth. If both of these conditions have been met, you may proceed. If not, see your instructor.
2. Using Controls #1 and #2 as comparisons, examine broths containing beads exposed to germicide (broths #1, #2, and #3). Using "G" to indicate growth and "NG" to indicate no growth; enter your results in both the individual data table and the class data table on the data sheet.
3. Again using Controls #1 and #2 as comparisons, examine Controls #3 and #4. Using "G" to indicate growth and "NG" to indicate no growth; enter your results on the data sheet.
4. Answer the questions on the data sheet.


1. Please read the caption for general instructions and advice.
2. Label one uninoculated NB tube "Control #1" and set it aside. (It is shown at the top right of this diagram).
3. Mix your original culture (labeled "OC" in the diagram at the lower left) until it is uniformly turbid. Then, transfer one loopful to control tube #2 (shown at the far right).
4. Using alcohol-flamed forceps, add four sterile beads to the original culture and let them soak for one minute.
5. After one minute of soaking, decant the nutrient broth into a beaker of disinfectant.
6. Using alcohol-flamed forceps, transfer the beads to the filter paper inside the 100 mm Petri dish to dry.
7a. When dry, transfer three beads to the three 60 mm Petri plates containing diluted germicide. Record the time.
7b. Then, transfer the fourth bead to a 60 mm plate containing sterile water.
7c. Lastly, transfer a sterile bead to the other 60 mm Petri plate containing sterile water.
8. After ten minutes from the recorded time, transfer the beads to their respective sterile broths.
9. Incubate all broths at 35°C for 48 hours.
OC
OC
Original culture (mix well)
Disinfectant (for broth disposal)
Sterile 100mm petri dish with filter paper
Dilution #1
Dilution #2
Dilution #3
Plate #4 Sterile water
Plate #5 Sterile water
Fifth (sterile) bead
Broth #1
Broth #2
Broth #3
Control #1
Control #2
Control #3
Control #4

2.54 Procedural Diagram for Chemical Germicides ♦
At first glance, this procedural diagram looks pretty imposing. Read it from left to right and follow the steps in sequence. With a little preparation and familiarity, it really isn't very complex.

References

McDonnell, Gerald E. *Antisepsis, Disinfection, and Sterilization: Types, Action, and Resistance*. Washington, DC: ASM Press, American Society for Microbiology, 2007.

Widmer, Andreas F. and Reno Frei. Chap. 7 in *Manual of Clinical Microbiology*, 9th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Marie Louise Landry, and Michael A. Pfaller, eds. Washington, DC: ASM Press, American Society for Microbiology, 2007.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 2-14

Chemical Germicides: Disinfectants and Antiseptics

OBSERVATIONS AND INTERPRETATIONS

1 Enter your individual data in the tables below.

Controls	Growth (G) or No Growth (NG)
#1	
#2	
#3	
#4	

Germicide/ Concentration	Growth (G) or No Growth (NG)
/	
/	
/	

2 Enter the class data in the table below. Use "G" for "Growth" and "NG" for "No Growth."

Organism	Household Bleach			Hydrogen Peroxide			Lysol® Brand II Disinfectant			Isopropyl Alcohol		
	0.01%	0.1%	1%	0.03%	0.3%	3%	25%	50%	100%	10%	30%	50%
S. epidermidis												
E. coli												

QUESTIONS

1 *Compare your results with the class data.*

a. *Which germicide was most effective and at what concentration? Defend your choice.*

b. *Which was least effective? Defend your choice.*

2

2 *Which organism seemed to be most resistant to the germicides?*

3 *Consider the controls.*

a. *Control #1: Was this a positive or a negative control? What purpose did it serve in the experiment?*

b. *Control #2: Was this a positive or a negative control? What purpose did it serve in the experiment?*

c. *Control #3: Was this a positive or a negative control? What purpose did it serve in the experiment?*

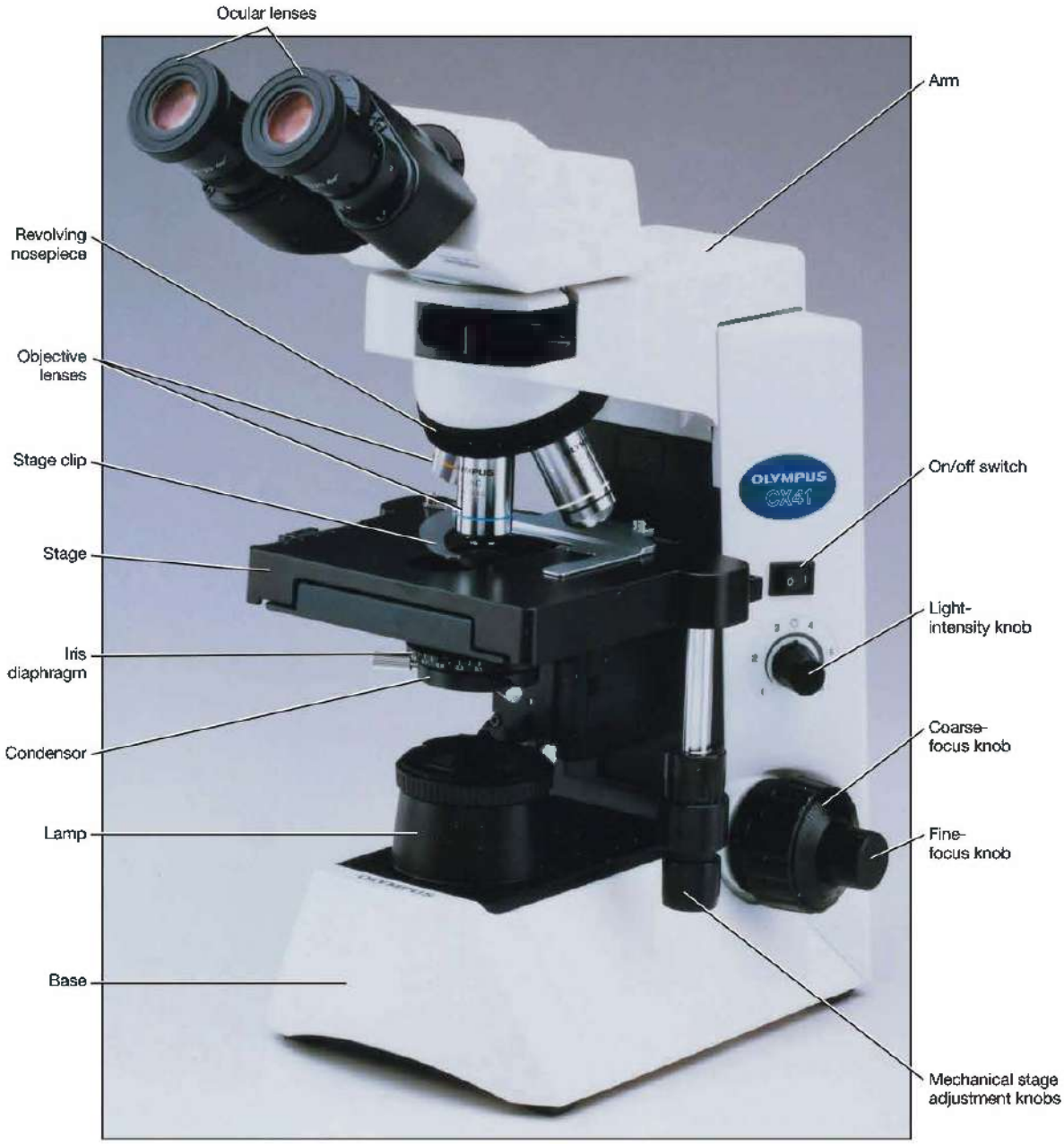
d. *Control #4: Was this a positive or a negative control? What purpose did it serve in the experiment?*

SECTION 3

Microscopy and Staining

Microbiology as a biological discipline would not be what it is today without microscopes and cytological stains. Our ability to visualize, sometimes in great detail, the form and structure of microbes too small or transparent to be seen otherwise is attributable to developments in microscopy and staining techniques. In this section you will learn (or refine) your microscope skills. Then you will learn simple and more sophisticated bacterial staining techniques.

The earliest microscopes used visible light to create images and were little more than magnifying glasses. Today, more sophisticated compound light microscopes (Fig. 3.1) are used routinely in microbiology laboratories. The various types of light microscopy include bright-field, dark-field, fluorescence, and phase contrast microscopy (Fig. 3.2). Although each method has specific applications and advantages, the one used most commonly in introductory classes and clinical laboratories is bright-field microscopy. Many research applications use electron microscopy because of its ability to produce higher-quality images of greater magnification.



3.1 Binocular Compound Microscope ◆ A quality microscope is an essential tool for microbiologists. Most are assembled with exchangeable component parts and can be customized to suit the user's specific needs.

Photograph courtesy of Olympus America Inc.

Introduction to the Light Microscope

EXERCISE 3-1

Theory

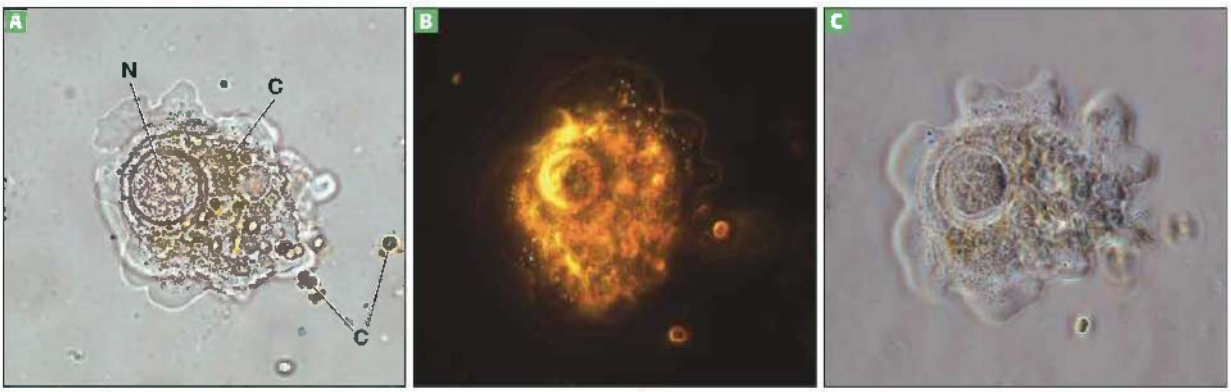
Bright-field microscopy produces an image made from light that is transmitted through a specimen (Fig. 3.2A). The specimen restricts light transmission and appears "shadowy" against a bright background (where light enters the microscope unimpeded). Because most biological specimens are transparent, the contrast between the specimen and the background can be improved with the application of stains to the specimen (Exercises 3-5 through 3-11 and 3-13). The "price" of the improved contrast is that the staining process usually kills cells. This is especially true of bacterial-staining protocols.

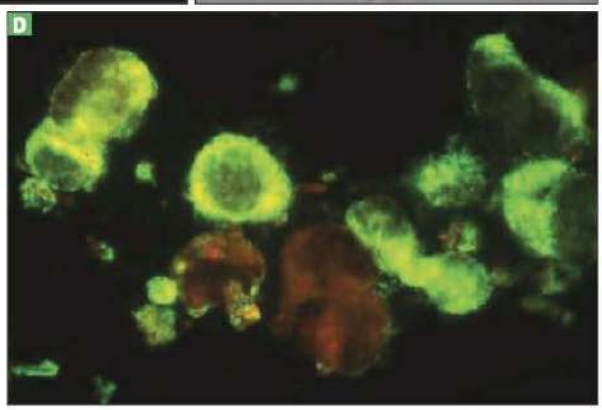
Image formation begins with light coming from an internal or an external light source (Fig. 3.3). It passes through the **condenser** lens, which concentrates the light and makes illumination of the specimen more uniform. **Refraction** (bending) of light as it passes through the **objective lens** from the specimen produces a magnified **real image.** This image is magnified again as it passes through the **ocular lens** to produce a **virtual image** that appears below or within the microscope. The amount of magnification that each lens produces is marked on the lens (Figs. 3.4A and 3.4B). Total magnification of the specimen can be calculated by using the following formula:

$$\text{Total Magnification} = \text{Magnification by the Objective Lens} \times \text{Magnification by the Ocular Lens}$$

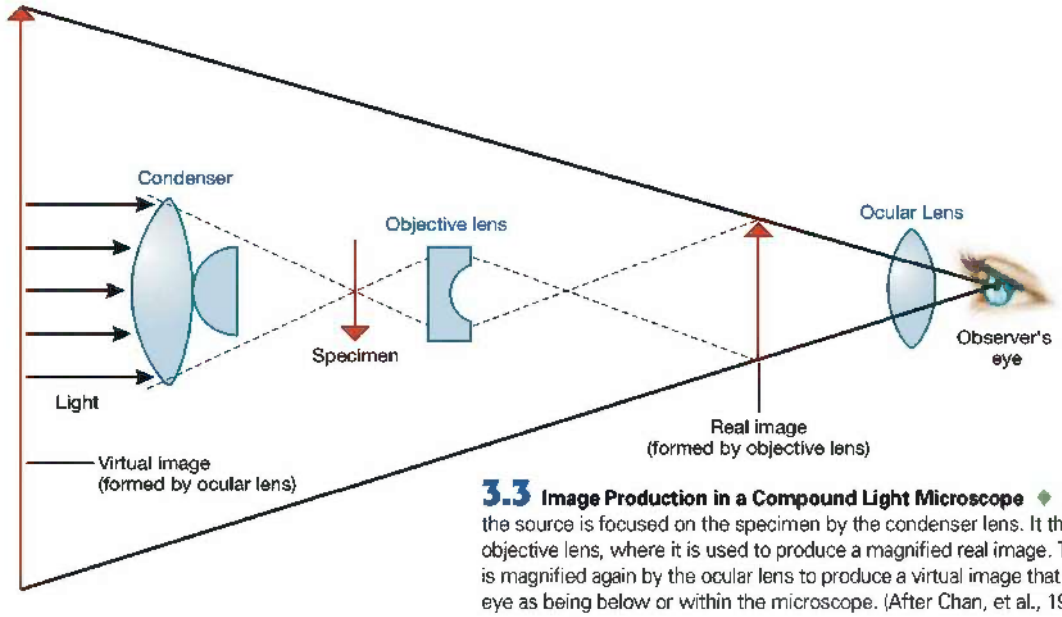
The practical limit to magnification with a light microscope is around 1300×. Although higher magnifications are possible, image clarity is more difficult to maintain as the magnification increases. Clarity of an image is called **resolution** (Fig. 3.5). The **limit of resolution** (or **resolving power**) is an actual measurement of how far apart two points must be for the microscope to view them as being separate. Notice that resolution *improves* as the limit of resolution is made *smaller*.

The best limit of resolution achieved by a light microscope is about 0.2 µm. (That is, at its absolute best, a light microscope cannot distinguish between two points





3.2 Types of Light Microscopy ◆ (A) This is a bright-field micrograph of an amoeba (called a "whole mount"). Because of its thickness, the entire organism is not in focus at once. Continually adjusting the fine focus to clearly observe different levels of the organism will give a sense of its three-dimensional structure. The nucleus (N) is obvious, as are the numerous cyanobacteria (C), both inside and outside the amoeba! Other granular material also is seen in the cytoplasm but notice its different texture toward the periphery. (B) This is a dark-field micrograph of the same amoeba. Notice the more three-dimensional image and that the peripheral cytoplasm is barely visible. (C) This is a phase contrast image of the same amoeba. Different parts of the interior and its detail are visible than what is seen in the other two micrographs. (D) This is a fluorescence micrograph of *Mycobacterium kansasii*. The apple green is one of the characteristic colors of fluorescence microscopy.



3.3 Image Production in a Compound Light Microscope ♦ Light from the source is focused on the specimen by the condenser lens. It then enters the objective lens, where it is used to produce a magnified real image. The real image is magnified again by the ocular lens to produce a virtual image that is seen by the eye as being below or within the microscope. (After Chan, et al., 1986)

3.4 Markings of Magnification and Numerical Aperture on Microscope Components ♦ (A) Three plan apochromatic objective lenses on the nosepiece of a light microscope. Plan means the lens produces a flat field of view. Apochromatic lenses are made in such a way that chromatic aberration is reduced to a minimum. From left to right, the lenses magnify 10×, 20×, and 40×, and have numerical apertures of 0.40, 0.70, and 0.85. The 20× lens has other markings on it. The mechanical tube length is the distance from the nosepiece to the ocular and has become standardized at 160 mm. However, this 20× lens has been corrected so the light rays are made parallel, effectively creating an infinitely long mechanical tube length (∞). This allows insertion of accessories into the light path without decreasing image quality. The thickness of cover glass to be used is also given (0.17 ± 0.01 mm). Also notice the standard colored rings for each objective: yellow for 10×, green for 20× (or 16×), and light blue for 40× (or 50×). (B) This is an oil-immersion lens. It is the only lens constructed in such a way as not to be damaged by oil and, as such, is the only one with which oil is to be used. Oil lenses are indicated by black-and-white rings. This particular lens is also constructed for phase contrast microscopy. It indicates it is to be used with the #3 setting on the phase condenser. (C) A 10× ocular lens. (D) A condenser (removed from the microscope) with a numerical aperture of 1.25. The lever at the right is used to open and close the iris diaphragm and adjust the amount of light entering the specimen.

closer together than 0.2 μm.) For a specific microscope, the actual limit of resolution can be calculated using the following formula:

$$D = \frac{\lambda}{NA_{condenser} + NA_{objective}}$$

where D is the minimum distance at which two points can be resolved, λ is the wavelength of light used, and $NA_{condenser}$ and $NA_{objective}$ are the numerical apertures of the condenser lens and objective lenses, respectively. Because numerical aperture has no units, the units for D are the same as the units for wavelength, which typically are in nanometers (nm).

Numerical aperture is the measure of a lens's ability to "capture" light coming from the specimen and use it to make the image. As with magnification, it is marked on the lens (Figs. 3.4A, 3.4B, and 3.4D). Using immersion oil between the specimen and the oil-immersion lens increases its numerical aperture and, in turn, makes its limit of resolution smaller. (If necessary, oil also may be placed between the condenser lens and the slide.) The result is better resolution.

3.5 Resolution and Limit of Resolution ◆ The headlights of most automobiles are around 1.5 m apart. As you look at the cars in the foreground of the photo, it is easy to see both headlights as separate objects. The automobiles in the distance appear smaller (but really aren't) as does the apparent distance between the headlights. When the apparent distance between automobile headlights reaches about 0.1 mm, they blur into one because that is the limit of resolution of the human eye.

The light microscope may be modified to improve its ability to produce images with contrast without staining, which often distorts or kills the specimen. In **dark-field microscopy** (Fig. 3.2B), a special condenser is used so only the light reflected off the specimen enters the objective. The appearance is of a brightly lit specimen against a dark background, and often with better resolution than that of the bright-field microscope.

Phase contrast microscopy (Fig. 3.2C) uses special optical components to exploit subtle differences in the refractive indices of water and cytoplasmic components to produce contrast. Light waves that are in phase (that is, their peaks and valleys exactly coincide) reinforce one another, and their total intensity (because of the summed amplitudes) increases. Light waves that are out of phase by exactly one-half wavelength cancel each other and result in no intensity—that is, darkness. Wavelengths that are out of phase by any amount will produce some degree of cancellation and result in brightness that is less than maximum but more than darkness. Thus, contrast is provided by differences in light intensity that result from differences in refractive indices in parts of the specimen that put light waves more or less out of phase. As a result, the specimen appears as various levels of "darks" against a bright background.

Fluorescence microscopy (Fig. 3.2D) uses a fluorescent dye that emits fluorescence when illuminated with ultraviolet radiation. In some cases, specimens possess naturally fluorescing chemicals and no dye is needed.

Application

Light microscopy (used in conjunction with cytological stains) is used to identify microbes from patient specimens or the environment. It also may be used to visually examine a specimen for the presence of more than one type of bacteria, or for the presence of other cell types that indicate tissue inflammation or contamination by a patient's cells.

In This Exercise

3

Today you will become familiar with the operation and limitations of your light microscope. You also will examine two practice slides to learn about microscope functioning.

✓ Materials

- ☐ Compound light microscope
- ☐ Lens paper
- ☐ Non-sterile cotton swabs
- ☐ Lens-cleaning solution or 95% ethanol
- ☐ Letter "e" slide
- ☐ Colored-threads slide

Instructions for Using the Microscope

Proper use of the microscope is essential for your success in microbiology. Fortunately, with practice and by following a few simple guidelines, you can achieve satisfactory results quickly. Because student labs may be supplied with a variety of microscopes, your instructor may supplement the following procedures and guidelines with instructions specific to your equipment. Refer to Figure 3.1 as you read the following (if working independently), or follow along on your microscope as your instructor guides you. (*Note:* This is a thorough treatment of microscope use, and not all parts may be immediately relevant to your laboratory. Refer back to this exercise as necessary.)

Transport

1. Carry your microscope to your workstation using both hands—one hand grasping the microscope's arm and the other supporting the microscope beneath its base.
2. Gently place the microscope on the table.

Cleaning

1. Lens paper is used for gently cleaning the condenser and objective lenses. Light wiping is usually enough. If that still doesn't clean the lens, call your instructor.
2. To clean an ocular, moisten a cotton swab with cleaning solution and gently wipe in a spiral motion starting at the center of the lens and working outward. Follow with a dry swab in the same pattern.

Basic Operation

1. Raise the substage condenser to a couple of millimeters below its maximum position nearly even with the stage (be sure not to raise it too high if you have already placed a slide on the stage) and open the iris diaphragm.
2. Plug in the microscope and turn on the lamp. Adjust the light intensity slowly to its maximum.
3. Using the nosepiece ring, move the scanning objective (usually 4×) or low-power objective (10×) into position. Do not rotate the nosepiece by the objectives because this can damage the objective lenses and cause them to unscrew from the nosepiece.
4. Place a slide on the stage in the mechanical slide holder and center the specimen over the opening in the stage.
5. If using a binocular microscope, adjust the distance between the two oculars as you examine the specimen to match your own interpupillary distance. Position your eyes above the oculars so the images from the two oculars fuse into one.
6. Adjust the iris diaphragm to produce optimum illumination, contrast, and image. In the simplest sense, this means opening the iris diaphragm as you increase magnification because a smaller portion of the light beam is entering the lens. (More specifically, use the maximum light intensity combined with the smallest aperture in the iris diaphragm that produces optimum illumination. Remember: This is bright-field microscopy, so don't close down the iris diaphragm too much unless necessary to see detail, as in unstained specimens.)
7. Use the coarse-focus adjustment knob to bring the image into focus. (*Note:* For most microscopes, the distance from the nosepiece opening to the focal plane of each lens has been standardized at 45 mm. This makes the lenses **parfocal** and gives the user an idea of where to begin focusing.) Bring the image into sharpest focus using the fine-focus adjustment knob. Then observe the specimen with your eyes relaxed and slightly above the oculars to allow the images to fuse into one. If you are using a monocular microscope, keep both eyes open anyway to reduce eye fatigue.
8. If you are using a binocular microscope, adjust the oculars' focus to compensate for differences in visual acuity of your two eyes. Close the eye with the adjustable ocular and bring the image into focus with the coarse- and fine-focus knobs. Then, using only the eye with the adjustable ocular, focus the image using the ocular's focus ring.
9. Scan the specimen to locate a promising region to examine in more detail. (*Note*: If your microscope has a field diaphragm, now would be a good time to set it up for Köhler illumination, which is described on page 147.)
10. If you are observing a nonbacterial specimen, progress through the objectives until you see the degree of structural detail necessary for your purposes. You will have to adjust the fine focus (but not the coarse focus because the lenses are parfocal—all that should be necessary after changing lenses is a slight adjustment) and illumination for each objective. Before advancing to the next objective, be sure to position a desirable portion of the specimen in the center of the field or you will risk "losing" it at the higher magnification.
11. If you are working with a bacterial smear, you will have to use the oil-immersion lens.
12. Follow these instructions to use the oil-immersion lens.
    - Work through the low (10×), then high-dry (40×) objectives, adjusting the fine focus and illumination for each. Before advancing to the next objective, be sure to position a desirable portion of the specimen in the center of the field or you risk "losing" it at the higher magnification.
    - When the specimen is in focus under high dry, rotate the nosepiece to a position midway between the high-dry and oil-immersion lenses. Then place a drop of immersion oil on the specimen. *Be careful not to get any oil on the microscope or its lenses, and be sure to clean it up if you do.* Rotate the oil lens so its tip is submerged in the oil drop, pass through it, and then return the oil lens into the oil. This minimizes the occurrence of air bubbles.
    - *Note*: Do not move the stage down to add oil to the slide or the specimen will no longer be in focus. On a properly adjusted microscope, the oil and the high-dry lenses have the same focal plane. Therefore, when a specimen is in focus on high dry, the oil lens, although longer, will also be in focus and won't touch the slide when rotated into position.)
    - Focus and adjust the illumination to maximize the image quality.
13. When you are finished, lower the stage (or raise the objective) and remove the slide. Dispose of the freshly prepared slides in a jar of disinfectant or a sharps container; return permanent slides to storage.

Köhler Illumination

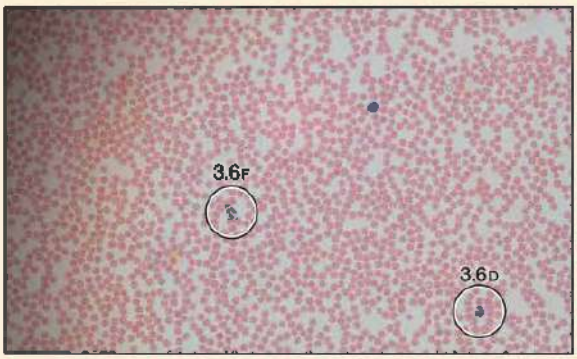
Köhler illumination was developed in 1893 by August Köhler while he was a graduate student in invertebrate zoology. It produces uniform and high-quality illumination of the specimen, but it requires components not found on all microscopes. If your microscope has a field diaphragm (Fig. 3.6A), you will need to adjust it for Köhler illumination to get the best image your microscope can produce.

1. Place a prepared slide, preferably a thin section of a specimen, but college microbiology labs may not have these readily available. For today's lab, you may use the letter "e" slide or another slide provided by your instructor. We have used a blood smear slide for illustration. After this lab, you will mostly be examining bacterial smears and you can use them. The specimen and field condenser may not be in focus.
2. Make sure the lamp is on. Open the field diaphragm to its maximum. Do the same with the iris diaphragm.
3. Bring the specimen into focus at low (10×) power (Fig. 3.6B). Adjust the light with the iris diaphragm if it is too bright.
4. Completely close the field diaphragm so you can see its edges (Fig. 3.6C). Depending on how out of adjustment your microscope is, the edges of the field diaphragm may only be a dark, blurry shadow, as in the lower left of Figure 3.6C or you may not be able to see them all. If you can't see the edges, you may need to raise the condenser (see step 5) and/or center the condenser using the centering screws on it (see step 6).
5. Adjust the height of the condenser until the edges of the field diaphragm come into focus (Fig. 3.6D). Should you see red and blue light fringes as you raise and lower the condenser, settle on the intermediate point between them.
6. Once the field diaphragm is in focus, you will need to center the condenser. Use the centering screws (Figs. 3.6E and 3.6F) located on the condenser to move it until the opening is nearly centered. Then, open the field diaphragm until it almost fills the field of view and do a final adjustment with the centering screws.
7. Open the field diaphragm until it just disappears from the field of view (Fig. 3.6G).
8. Adjust the light with the iris diaphragm so the specimen is optimally illuminated (Fig. 3.6H).
9. You will need to make these adjustments for each objective lens. However, when examining bacterial smears you will be using the lower-power objectives only to focus and center the specimen; only the oil-immersion lens will be used for serious examination. This means that once you set up the oil lens for Köhler illumination, you can leave it there. High-quality illumination is not needed for the lower powers.

Köhler Illumination

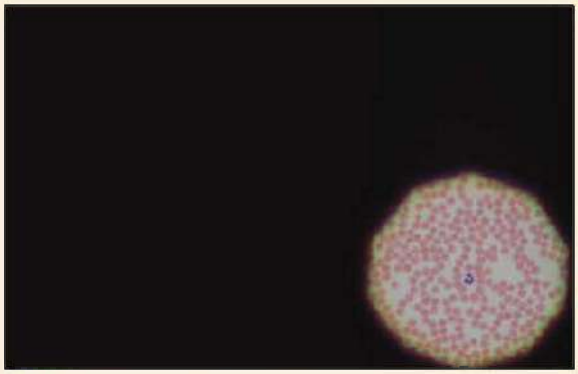
Köhler illumination allows focusing of the light source on the specimen, which produces more uniform illumination. All photos in this sequence were shot with the same microscope light intensity. The only adjustments made are those written in the captions as part of the process. ◆

3.6A The Field Diaphragm ◆ The field diaphragm is located in the microscope's base. It can be adjusted by rotating the ring around the lamp (arrow).

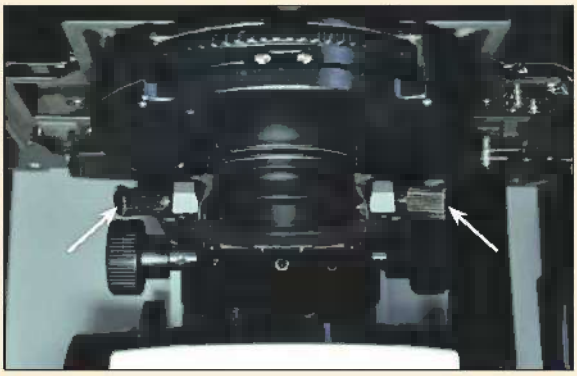


3.6B Bring the Specimen into Focus ◆ Notice the uneven illumination of this field. It is darker on the left than on the right, which means it needs adjusting. For perspective, the circled white blood cells are the ones seen in Figures 3.6D and 3.6F.

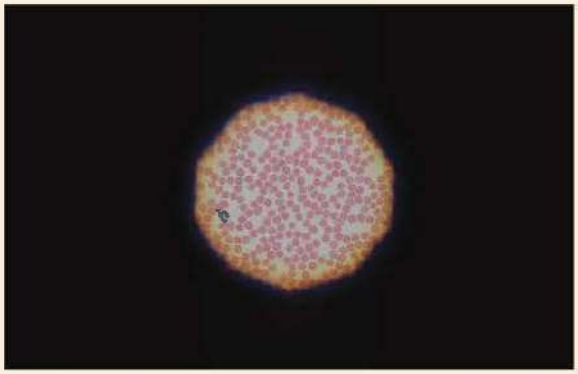
3.6C Close the Field Diaphragm ◆ Closing the field diaphragm has cut down the amount of light entering the objective lens, making the image dark. The actual light intensity setting on the microscope is the same in this micrograph as in all the others (except Fig. 3.6H).



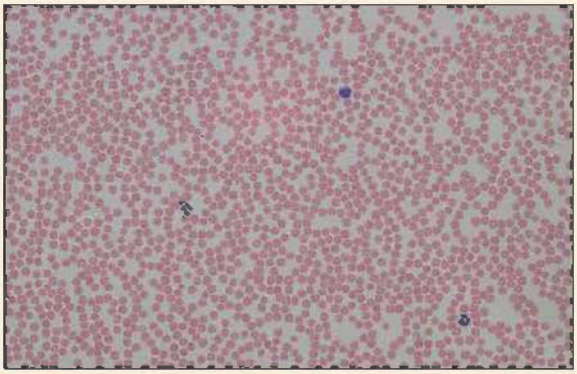
3.6D Adjust the Condenser Height until the Edges of the Field Diaphragm are in Focus ◆ What was barely visible in Figure 3.6C now is illuminated at the same level as in Figure 3.6B and is still in focus.



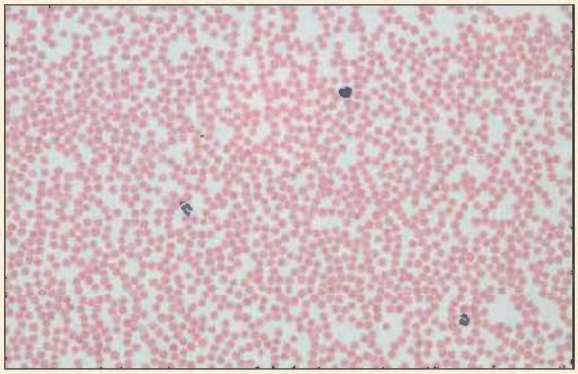
3.6E Use the Condenser Centering Screws (Arrows) to Center the Light in the Field of View ◆ This may take some practice, because they apply and release pressure on the condenser to move it in different directions.



3.6F Center the Field Diaphragm ◆ Notice that it is the lighted area that has moved, not the slide itself. Compare this lighted area with Figure 3.6B.



3.6G Open the Field Diaphragm until It Just Goes Out of View ◆ Readjust the centering if it is a little off.



3.6H Adjust the Light With the Iris Diaphragm ◆ For bright-field microscopy, the background should be white, but not so bright as to lose detail. Note the more uniform illumination compared to Figure 3.6B.

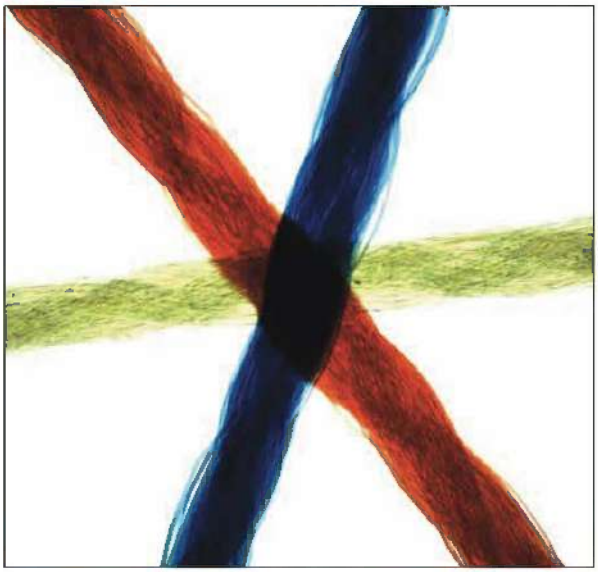
Storage

When you are finished for the day:

1. Move the scanning objective into position.
2. Center and lower the mechanical stage.
3. Lower the light intensity to its minimum, and then turn off the light.
4. Wrap the electrical cord according to your particular lab rules.
5. Clean any oil off the lenses, stage, etc. Be sure to use only cotton swabs or lens paper for cleaning any of the optical surfaces of the microscope (see "Cleaning," page 145).
6. Return the microscope to its appropriate storage place.

Procedure

1. Get out your microscope and record the magnifications and numerical aperture values in the chart on the data sheet, page 151.
2. Clean your microscope lenses as outlined in "Instructions for Using the Microscope."
3. Plug in the microscope and position the scanning objective over the stage. Make condenser and lamp adjustments appropriate for scanning power.
4. Pick up the letter "e" slide and examine it without the microscope. Record the orientation of the letter when the slide label is on the left. Sketch the orientation of the letter "e" on your data sheet.
5. Place the slide on the stage in the same position as you examined it with your naked eyes. Now, center the "e" in the field and examine it with the scanning objective. After focusing, sketch the orientation of the letter "e" image as viewed with the microscope on your data sheet. (If your microscope has a field condenser, you should adjust it for Köhler illumination after focusing.)
6. Now, move the *stage* to the right and record the direction the *image* moves.[1]
7. Position the "e" in the center of the field again. Move the *stage* toward you and record on your data sheet the direction the *image* moves. Then remove the slide from the microscope.
8. Examine the colored-threads slide without the microscope (Fig. 3.7). See if you can tell where in the stack of three threads each color resides. That is, is the red thread on the top, bottom, or middle? Do the same for the yellow and blue threads. Record your observations on the data sheet.
9. Now, place the slide on the microscope and determine the order of the threads using the low- and high-power objectives. Record your observations on the data sheet.



3.7 Challenge of the Threads ◆ Even with the microscope, determining the order of threads from top to bottom is a challenge. This will require patience and use of the fine focus! Making it worse, not all the slides will be the same. Good luck!

References

Abramowitz, Mortimer. *Microscope Basics and Beyond.* Olympus America Inc. Melville, NY: Scientific Equipment Group, 2003.

Ash, Lawrence R. and Thomas C. Orihel. Pages 187–190 in *Parasites: A Guide to Laboratory Procedures and Identification.* Chicago: American Society for Clinical Pathology (ASCP) Press, 1991.

Bradbury, Savile and Brian Bracegirdle. Chap. 1 in *Introduction to Light Microscopy.* Oxford, UK: BIOS Scientific Publishers Limited, 1998.

Forbes, Betty A., Daniel F. Sahm, and Alice S. Weissfeld. Pages 119–121 in *Bailey & Scott's Diagnostic Microbiology,* 11th ed. St. Louis, MO: Mosby, 2002.

[1] If your microscope doesn't have a mechanical stage, move the slide with your hands in the appropriate direction.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-1

Introduction to the Light Microscope

DATA AND CALCULATIONS

1 Record the relevant values off your microscope and perform the calculations of total magnification for each lens.

Lens System	Magnification of Objective Lens	Magnification of Ocular Lens	Total Magnification	Numerical Aperture	Calibration of Ocular Micrometer
Scanning					
Low power					
High dry					
Oil immersion					
Condensor lens	NA	NA	NA		NA

2 Sketch your observations of the letter "e" slide in the table below. Be sure the slide is right side up with the label at the left.

Appearance of the "e" with the Naked Eye	Appearance of the "e" Under the Microscope	When the stage moves to the right, the image moves to the...	When the stage moves toward you, the image moves...

3 Record your observations of the colored-threads slide below. Check it under low and high power and see if your answer changes.

Low power (usually the 10× objective)	Top:
	Middle:
	Bottom:
High power (usually the 40× objective)	Top:
	Middle:
	Bottom:

QUESTIONS

1 *Why aren't the magnifications of both ocular lenses of a binocular microscope used to calculate total magnification?*

3

2 *What is the total magnification for each lens setting on a microscope with 15× oculars and 4×, 10×, 45×, and 97× objectives lenses?*

3 *Assuming that all other variables remain constant, explain why light of shorter wavelengths will produce a clearer image than light of longer wavelengths.*

4 *Why is wavelength the main limiting factor on limit of resolution in light microscopy?*

5 *On a given microscope, the numerical apertures of the condenser and low-power objective lenses are 1.25 and 0.25, respectively. You are supplied with a filter that selects a wavelength of 520 nm.*

a. *What is the limit of resolution on this microscope?*

b. *Will you be able to distinguish two points that are 300 nm apart as being separate, or will they blur into one?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-1

(continued)

6 *On the same microscope as in question 5, the high-dry objective lens has a numerical aperture of 0.85.*

a. *What is the limit of resolution on this microscope?*

b. *Will you be able to distinguish two points that are 300 nm apart as being separate, or will they blur into one?*

7 *Calculate the limit of resolution for the oil lens of your microscope. Assume an average wavelength of 500 nm.*

8 *Examine Figure 3.3 and explain the results you observed with the letter "e" slide.*

9 *With which objective was it easier to determine the sequence of colored threads?*

10 *Why should closing the iris diaphragm improve your ability to determine thread order?*

11 *What does the colored-threads slide demonstrate about specimens you will be observing later in the class?*

Calibration of the Ocular Micrometer

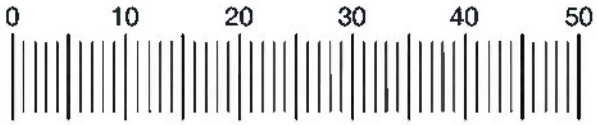
EXERCISE 3-2

Theory

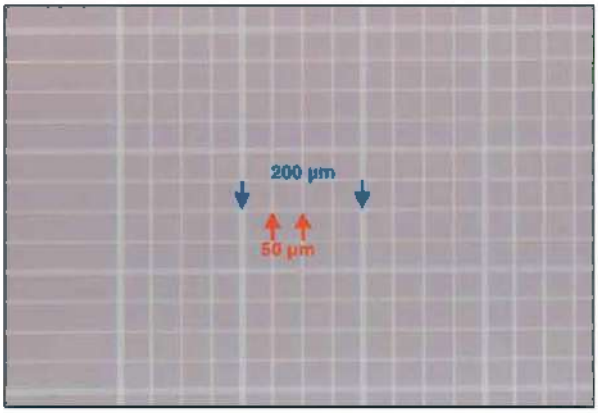
An **ocular micrometer** is a type of ruler installed in the microscope eyepiece, composed of uniform but unspecified graduations (Fig. 3.8). As such, it must be calibrated before any viewed specimens can be measured. The device used to calibrate ocular micrometers is called a **stage micrometer.** As illustrated in Figure 3.9, a stage micrometer is a type of microscope slide containing a ruler with 10 µm and 100 µm graduations. Other measuring instruments may be used in place of a stage micrometer, as shown in Figure 3.10.

When the stage micrometer is placed on the stage, it is magnified by the objective being used; therefore, the size of the graduations (relative to the ocular micrometer divisions) increases as magnification increases. Consequently, the *value* of ocular micrometer divisions decreases as magnification increases. For this reason, calibration must be done for each magnification.

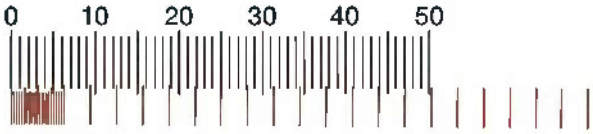
As shown in Figure 3.11, the stage micrometer is placed on the stage and brought into focus such that it is superimposed by the ocular micrometer. Then the first (left) line of the ocular micrometer is aligned with one of



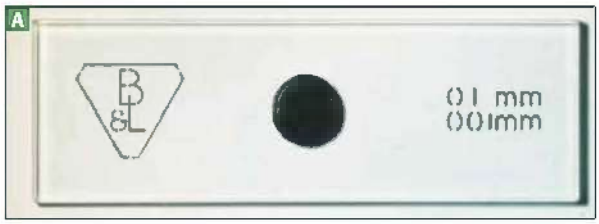
3.8 Ocular Micrometer ◆ This illustration representing an ocular micrometer shows a scale with uniform increments of unknown size. It has to be calibrated for each objective lens on the microscope.



3.10 Hemacytometer ◆ Any instrument with markings of known distance apart may be used as a stage micrometer. The hemacytometer is a grid with lines 50 µm apart (red arrows). A larger grid is formed by lines 200 µm apart (blue arrows). Use any horizontal line as the micrometer, with the smallest divisions 50 µm apart.



3.11 What They Look Like in Use ◆ When properly aligned, the ocular micrometer scale is superimposed over the stage micrometer scale. Notice that they line up at their left ends. These two micrometers have intentionally not been drawn to the correct proportions relative to each other because they are intended only to show how to use them. When you work with your microscope and stage micrometer, expect different numbers to align.



3.9 Stage Micrometer ◆ (A) The stage micrometer is a microscope slide with a microscopic ruler engraved into it (not visible in the dark center of the slide). The markings on this micrometer indicate that the major increments are 0.1 mm (100 µm) apart. There is also a section of the scale that is marked off in 0.01 mm (10 µm) increments. (B) This drawing represents what the stage micrometer on the slide in A looks like. The micrometer is 2,200 µm long. The major divisions are 100 µm apart. The 200 µm at the left are divided into 10 µm increments.

the marks on the stage micrometer. (The line chosen on the stage micrometer depends on the power of the lens being calibrated. Lower powers use the large graduations; higher powers use the smaller graduations on the left. Figure 3.11 illustrates proper alignment with the scanning objective.)

Notice in Figure 3.11 that line 25 of the ocular micrometer and the eighth major line of the stage micrometer are perfectly aligned. (*Note*: This is a hypothetical situation. Your stage and ocular micrometers won't align in the same way.) This indicates that 25 ocular micrometer divisions (also called ocular units, or OU) span a distance of 800 µm because the stage micrometer lines are 100 µm apart. Notice also that line 47 of the ocular micrometer is aligned with the fifteenth major stage micrometer line. This means that 47 ocular units span 1,500 µm. These values have been entered for you in Table 3-1.

To determine the value of an ocular unit on a given magnification, divide the distance (from the stage micrometer) by the corresponding number of ocular units.

$$\frac{800\ \mu m}{25\ \text{ocular units}} = 32\frac{\mu m}{OU}$$

$$\frac{1{,}500\ \mu m}{47\ \text{ocular units}} = 31.9\frac{\mu m}{OU} \approx 32\frac{\mu m}{OU}$$

As shown in Table 3-1, it is customary to record more than one measurement. Each measurement is calculated separately. If the calculated ocular unit values differ, use their arithmetic mean as the calibration for that objective lens.

TABLE 3-1 Sample Data from Figure 3.11

Stage Micrometer	Ocular Micrometer	Calibration
800 µm	25 OU	$\frac{32\ \mu m}{OU}$
1,500 µm	47 OU	$\frac{31.9\ \mu m}{OU} \approx \frac{32\ \mu m}{OU}$

As mentioned previously, each magnification must be calibrated. Because of its short working distance, calibrating the oil-immersion lens may be difficult to accomplish using the stage micrometer. It also may be difficult because the distance between stage micrometer lines is too large. If this is the case, its value can be calculated using the calibration value of one of the other lenses. Refer to Table 3-2 for the total magnifications for each objective lens on a typical microscope. Notice that the magnification of the oil-immersion lens is 10 times greater than the low-power lens. This means that objects viewed on the stage (stage micrometer *or* specimens) appear 10 times larger when changing from low power to oil immersion. But, because the magnification of the ocular micrometer does not change, an ocular division now covers only one-tenth the distance. Thus, the size of an ocular unit using the oil-immersion lens can be calculated by dividing the calibration for low power by 10.

Ocular micrometer values can be calculated for any lens using values from any other lens, and they provide a good check of measured values. As calculated from Figure 3.11, 32 µm/OU was the calibration for the scanning objective of a hypothetical microscope. For practice, calculate the low, high-dry and oil-immersion calibrations. Write the values in Table 3-2.

You are given the calibration of the ocular micrometer of the scanning objective for the hypothetical microscope as calculated in the text (Table 3-1). You are also given the total magnifications produced when using the other objectives. Use this information to calculate the remaining calibrations as described in the text.

TABLE 3-2 Using one Calibration to Calculate the Others—A Sample Problem

Power	Total Magnification	Calibration (µm/OU)
Scanning	40×	32
Low Power	100×	
High-Dry Power	400×	
Oil Immersion	1000×	

Once you have determined the ocular unit values for each objective lens on your microscope, use the ocular micrometer as a ruler to measure specimens. For instance, if you determine that under the scanning objective a cell is 5 ocular units long, the cell's actual length would be determined as follows (using the sample values from Table 3-2):

Cell Dimension = Ocular Units × Calibration
Cell Dimension = 5 Ocular Units × 32 µm/OU
Cell Dimension = 160 µm

Be sure to include the proper units in your answer!

Application

The ability to measure microbes is useful in their identification and characterization.

In This Exercise

This lab exercise involves calibrating the ocular micrometer on your microscope. Actual measurement of specimens will be done in subsequent lab exercises as assigned by your instructor.

✓ Materials

Per Student

- ☐ Compound microscope equipped with an ocular micrometer
- ☐ Stage micrometer

Procedure

Following is the general procedure for calibrating the ocular micrometer on your microscope. Your instructor will notify you of any specific details unique to your laboratory.

1. Check your microscope and determine which ocular has the micrometer in it.
2. Move the scanning objective into position.
3. Place the stage micrometer on the stage and position it so its image is superimposed by the ocular micrometer and the left-hand marks line up.
4. Examine the two micrometers and, as previously described, record two or three points where they line up exactly. Record these values on the data sheet, page 159, and calculate the value of each ocular unit.
5. Change to low power and repeat the process.
6. Change to high-dry power and repeat the process.
7. Change to the oil-immersion lens and repeat the process. Be sure to look at the oil lens from the side as you rotate it into position. If it looks like it is going to contact the stage micrometer, **stop!** Return the high-dry lens into position and complete the calibration from the value of another lens. You will also need to calculate the oil lens' calibration if the stage micrometer lines are too far apart for direct measurement.
8. Compute average calibrations for each objective lens and record these on the data sheet.
9. As long as you keep this microscope throughout the term, you may use the calibrations you recorded without recalibrating the microscope.

References

Abramoff, Peter and Robert G. Thompson. Pages 5–6 in *Laboratory Outlines in Biology—III*. San Francisco: W. H. Freeman, 1982.

Ash, Lawrence R. and Thomas C. Orihel. Pages 187–190 in *Parasites: A Guide to Laboratory Procedures and Identification*. Chicago: American Society for Clinical Pathology (ASCP) Press, 1991.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-2

Calibration of the Ocular Micrometer

DATA AND CALCULATIONS

1 Record two or three values where the ocular micrometer and the stage micrometer line up for the scanning, low, high-dry, and oil-immersion objective lenses. Then calculate the calibration for each. Be sure to include proper units in your calibrations.

Scanning Objective Lens

Stage Micrometer (µm)	Ocular Micrometer (OU)	Calibration

Low-Power Objective Lens

Stage Micrometer (µm)	Ocular Micrometer (OU)	Calibration

High-Dry Objective Lens

Stage Micrometer (µm)	Ocular Micrometer (OU)	Calibration

3

Oil-Immersion Objective Lens

Stage Micrometer (µm)	Ocular Micrometer (OU)	Calibration

2 Calculate the average value for each calibration and record them in the table below. If necessary, use one of the values to calculate the calibration of the oil lens. Be sure to include proper units in your answer.

Average Calibrations for My Microscope

Objective Lens	Average Calibration
Scanning	
Lower power	
High-dry power	
Oil immersion	

Microscopic Examination of Eukaryotic Microbes

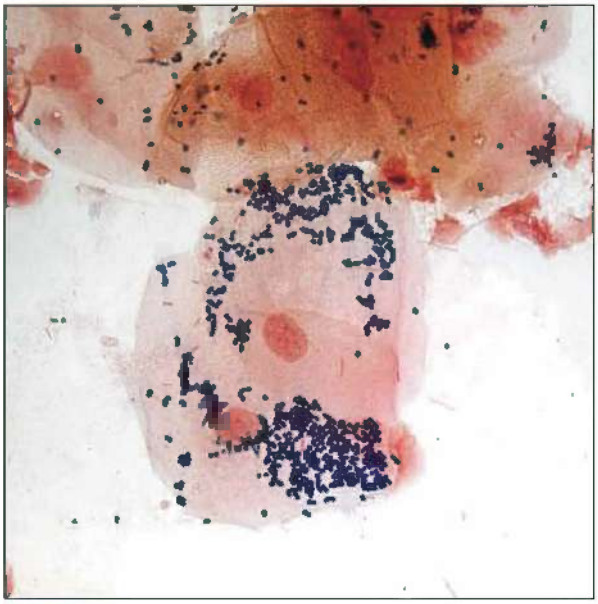
EXERCISE 3-3

Theory

Cells are divided into two major groups—the **prokaryotes**[1] and the **eukaryotes**—based on size and complexity. These and other differences are summarized in Table 3-3 and shown in Figure 3.12. The prokaryotes are further divided into two domains—the *Archaea* and the *Bacteria*. The eukaryotes belong to a single domain and are divided into four "supergroups" until kingdom associations can reasonably be determined. Figure 3.13 provides a phylogenetic tree of these groups based on RNA comparisons and other data.

In this lab you will observe representative simple eukaryotes from the four supergroups. Some specimens are on commercially prepared slides, whereas others need to be prepared as wet mounts (Fig. 3.14) of living cultures. In a wet mount, a drop of water is placed on the slide and the organisms are introduced into it. Or, if the organism is already in a liquid medium, then a drop of medium is placed on the slide. A cover glass is placed over the preparation to flatten the drop and keep the objective lens from getting wet. A stain may or may not be applied to add contrast.

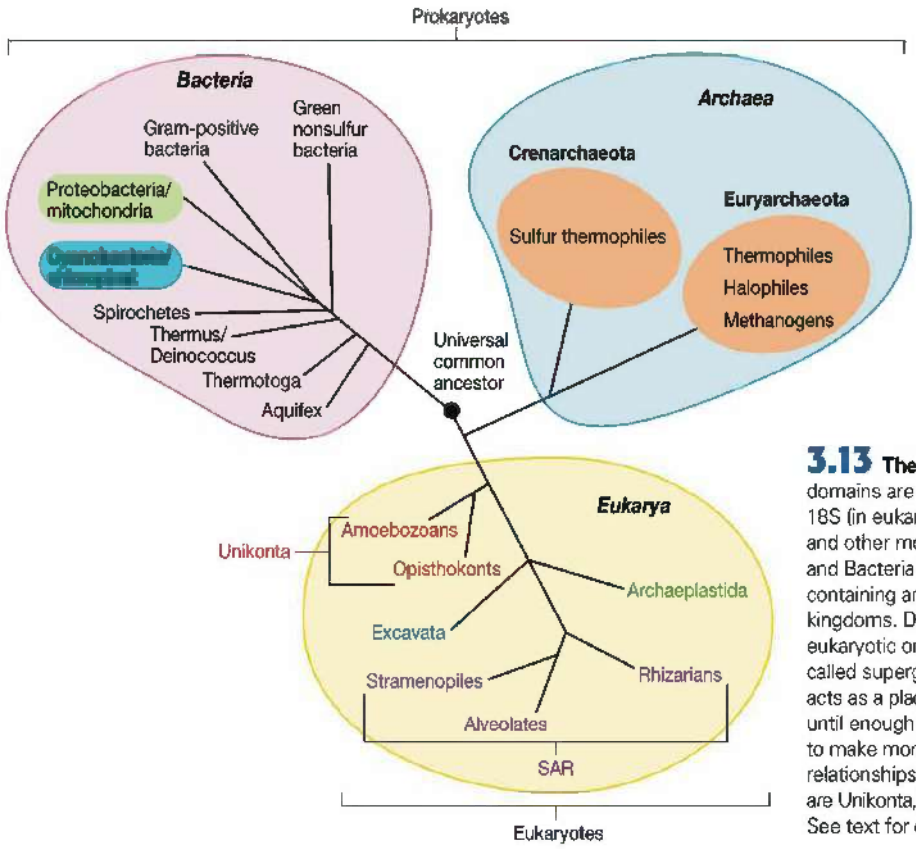
[1] The validity of the term *prokaryote* has recently been called into question. We continue to use it because of the uncertainty of the change.



3.12 Prokaryotic and Eukaryotic Cells (Gram Stain, Oil Immersion) ◆ This is a direct smear specimen taken from around the base of the teeth below the gum line. The large, pink cells are human epithelial cells and are eukaryotic (notice the prominent nuclei). The small, purple cells are prokaryotic bacteria. Typically, prokaryotic cells range in size from 1 µm to 5 µm, whereas eukaryotic cells are in the 10 µm to 100 µm range.

TABLE 3-3 Summary of Major Prokaryotic and Eukaryotic Features

Character	Prokaryotes	Eukaryotes
Organismal groups	*Bacteria* and *Archaea*	Plants, animals, fungi, and simple eukaryotes
Typical size	1 µm–5 µm	10 µm–100 µm
Membrane-bound organelles (including a nucleus)	Absent	Present
Ribosomes	70S (30S and 50S subunits)	80S (40S and 60S subunits)
Microtubules	Absent	Present
Flagellar movement	Rotary	Whiplike
DNA	Single, circular, double-stranded DNA (dsDNA) molecule called a chromosome	Two to many linear dsDNA molecules; each is a chromosome
Introns	Absent (sometimes in *Archaea*)	Common
Mitotic division	Absent	Present



3.13 The Three Domains of Life ♦ The domains are based on 16S (in prokaryotes) and 18S (in eukaryotes) rRNA sequencing results and other molecular comparisons. The Archaea and Bacteria are prokaryotic domains, each containing an as yet undetermined number of kingdoms. Domain Eukarya includes all the eukaryotic organisms and is divided into so-called supergroups, an informal category that acts as a place to temporarily group organisms until enough evidence has been accumulated to make more informed decisions about their relationships and placement. The supergroups are Unikonta, Excavata, SAR, and Archaeplastida. See text for details.

1. If observing a microbe obtained from a solid medium, place a loopful or a drop of water on a clean glass slide. If the specimen is already in a liquid medium, this step is unnecessary.

2. Use an inoculating needle or an inoculating loop to transfer the specimen from a solid medium into the water and gently mix. If the specimen is in a liquid medium, simply transfer a loopful or a drop of the specimen to the clean glass slide.

3. Gently lower the cover glass onto the drop with your index finger and thumb, or use a loop. Be careful not to trap bubbles.

If not staining...

4. Observe under the microscope, but do not use the oil-immersion lens because it may hit the cover glass.

If staining...

4. Add a drop or two of stain next to the cover glass. Draw the stain under the cover glass with a piece of paper in contact with the cover glass edge on opposite side.

5. Observe under the microscope, but do not use the oil-immersion lens because it may hit the cover glass.

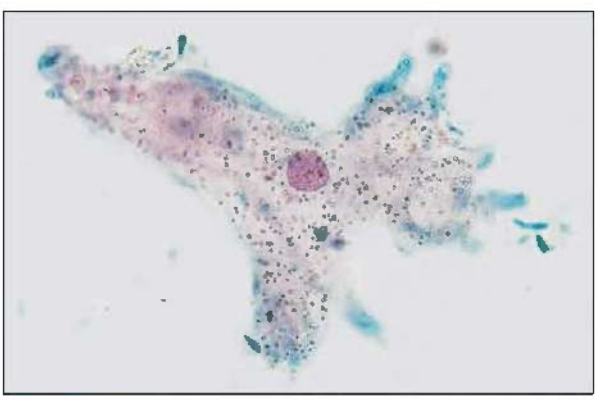
3.14 Wet Mount Procedural Diagram ♦ Use these instructions to make wet mounts of living specimens. The preparation may be stained or not, but staining will eventually kill the organisms. Of course, so will drying. You can expect a wet mount to last only 15–20 minutes.

Eukaryotic Supergroups

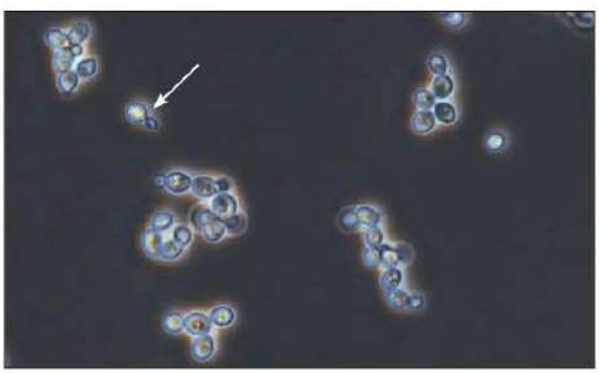
Only a brief introduction to the eukaryotic supergroups (Excavata, SAR [formerly Chromalveolata and Rhizaria], Archaeplastida, and Unikonta) will be given here, with an emphasis on microscopic and nonpathogenic species. A sampling of pathogenic species is covered in Section 12.

- **Supergroup Unikonta.** This group is composed of heterotrophs and includes amoebas (of all sorts), animals, fungi, and others. There is evidence that unikonts diverged from the other eukaryotic lineages fairly early in eukaryote evolution. This is represented by their branch point being closer to the "root" of the phylogenetic tree in Figure 3.13.
  - Amoebas belong to two main groups. "Classical" amoebas belong to a subgroup called **Gymnamoebas** (Fig. 3.15). These are free-living in marine, freshwater, and soil environments. The other group comprises the **entamoebas**, which are parasitic and are covered in Exercise 12-3. All amoebas move by forming cytoplasmic extensions called **pseudopods**. They also engulf their food with them. Division is by binary fission and they do not have a cell wall.
  - Fungi are nonmotile eukaryotes. Their cell wall is usually made of the polysaccharide **chitin**, not cellulose as in plants. Unlike animals and many other unikonts (that ingest then digest their food), fungi are **absorptive heterotrophs**. That is, they secrete **exoenzymes** into the environment, and then absorb the digested nutrients. Most are saprophytes that decompose dead organic matter, but some are parasites of plants, animals, or humans. Fungi are informally divided into **unicellular yeasts** and **filamentous molds** based on their overall appearance. Molds and parasitic yeasts are covered in Exercise 12-1.

    Saccharomyces cerevisiae (Fig. 3.16) is the common brewer's yeast and is used in production of bread, wine, and beer, but is not an important human pathogen. Asexual reproduction occurs by uneven cell division called **budding**. Sexual reproduction involves meiosis of the yeast cell nucleus to produce haploid spores that can combine to form new diploid cells.
- **Supergroup Excavata.** These are unicellular species that usually have a feeding groove *excavated* from one side of the cell and possess one or more flagella. Many species have a life cycle in which there is an active feeding stage called the **trophozoite** and an inert resting stage called a **cyst**. Four subgroups are recognized: **parabasalids, diplomonads, kinetoplastids,** and **euglenozoans**. Only the euglenozoans are considered here. The other three are covered in Exercise 12-3 (Figs. 12.29 through 12.32).
  - Euglenozoans are unicellular, green, photosynthetic autotrophs when light is available, but can live heterotrophically when it is not (making them **mixotrophic**). They usually have two flagella, or more rarely only one. The red "eyespot" is a distinctive characteristic that is easily seen with the light microscope. A euglenid is shown in Figure 3.17.
- **Supergroup Archaeplastida.** There is strong evidence to support the conclusion that members of this group are all descended from an ancestor that engulfed a cyanobacterium (by **primary endosymbiosis**), which ultimately evolved into the chloroplasts of green algae, red algae, and true plants. Thus, all members are autotrophic. Most also possess walls made of cellulose. Two groups are covered here: **chlorophytes** and **charophytes**.

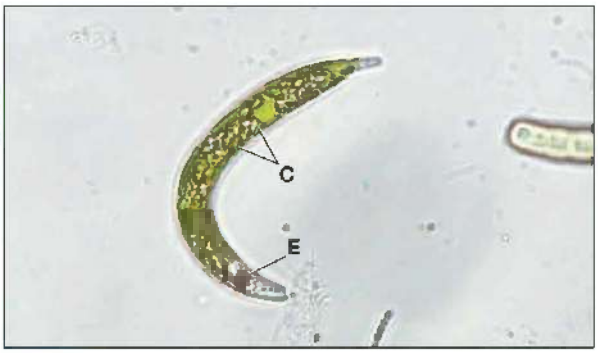


3.15 Supergroup Unikonta (Gymnamoeba Subgroup) ◆
This *Amoeba* has numerous pseudopods, which are used for movement as well as engulfing prey. Also see Figures 3.2A through 3.2C.



3.16 Supergroup Unikonta (Fungus Subgroup–Yeasts) ◆
The brewer's yeast *Saccharomyces cerevisiae* is shown in a wet-mount preparation using phase contrast microscopy. The cells are oval with dimensions of 3–8 µm by 5–10 µm. One budding cell is indicated by the arrow.

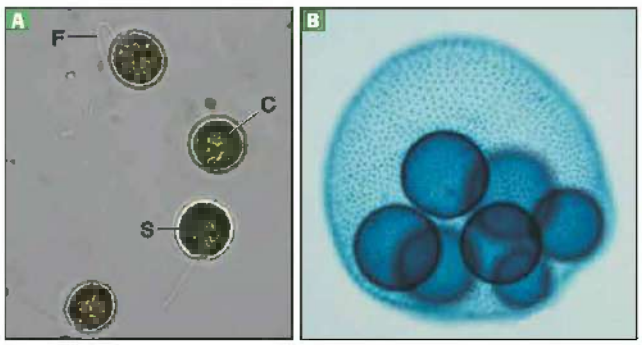
- The chlorophytes are commonly called green algae. Most are freshwater, unicellular, filamentous, or colonial species. Flagella are commonly used for motility, as in the haploid, unicellular *Chlamydomonas* (Fig. 3.18A). *Volvox* (Fig. 3.18B) is an example of a colonial species made of *Chlamydomonas*-like cells. *Volvox* colonies can undergo sexual reproduction; there are male colonies that produce sperm bundles and female colonies that produce eggs. They also can undergo asexual reproduction by forming daughter colonies within, which are ultimately released as new individuals.
- Closely related to the chlorophytes, but even more closely related to true plants (because of certain aspects of cell ultrastructure and physiology), are the charophytes, which include *Spirogyra* (Fig. 3.19).



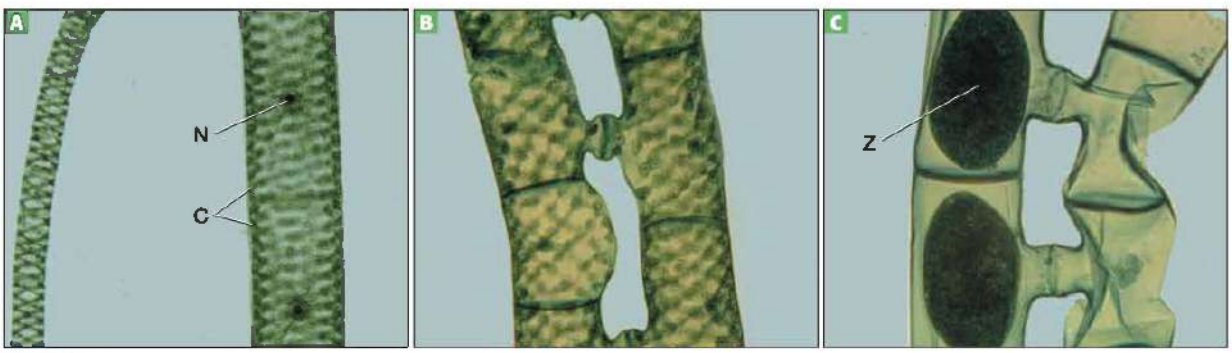
3.17 Supergroup Excavata (Subgroup Euglenozoa) ♦ *Euglena* is a large genus of mixotrophic flagellates. Most species have chloroplasts (C), which are discoid in this specimen. A red "eyespot" (E) is located in the colorless anterior of the cell. The single flagellum also emerges from the anterior.

- **SAR Supergroup.** This is a very diverse group recently joined primarily because of strong similarities in DNA sequences of the entire genome. Subsumed into the SAR supergroup are stramenopiles, alveolates, and rhizarians (from which the acronym SAR is derived). Only stramenopiles and alveolates are considered here.

The main unifying feature of stramenopiles and alveolates (though not demonstrated in all groups) is evidence of being descendants of an ancestor that engulfed a red algal cell that evolved into a plastid with red algal characteristics. (Because the red alga already



3.18 Supergroup Archaeplastida (Chlorophyte Subgroup) ♦ (A) *Chlamydomonas* is a unicellular green alga and a single, cup-shaped chloroplast (C). A red-orange stigma (S) is found within the chloroplast and is involved in phototaxis (attraction to light). It also has two flagella (F) for locomotion. Cells are approximately 30 µm long and 20 µm wide. (B) Volvox is a spherical colonial green alga made of cells similar in shape to *Chlamydomonas*. In this preserved specimen, asexually produced daughter colonies are visible within the parent colony. Daughter colonies evert as they are released from the parent colony. Mature colonies can reach a size visible to the naked eye.



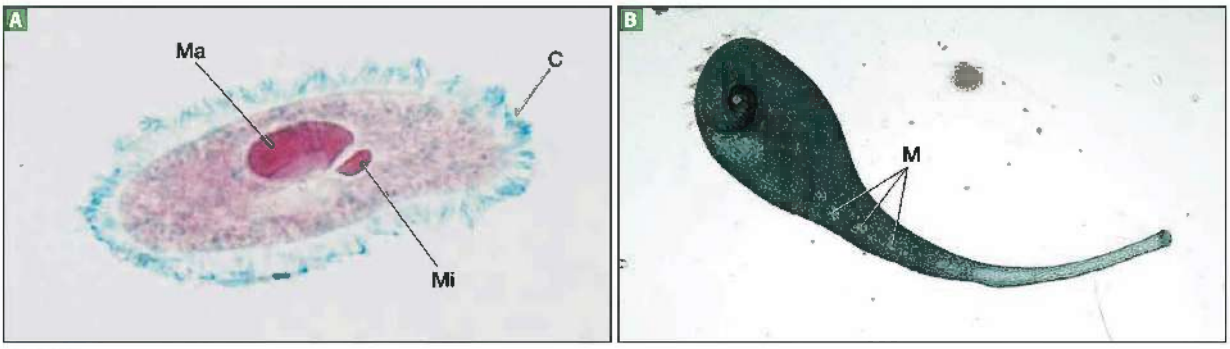
3.19 Supergroup Archaeplastida (Charophyte Subgroup) ♦ *Spirogyra* is a filamentous alga with distinctive spiral chloroplasts (C). The cells are haploid, that is, they have only one complete set of chromosomes. (A) Two vegetative filaments are shown here. Also note the nuclei (N) in the cells on the right. (B) These two filaments have begun **scalariform conjugation**, a sexual process that occurs between different filaments. The protrusions are called **conjugation tubes** and serve as a connection through which the cytoplasm of one cell (acting as a gamete) can be moved and join with the cytoplasm of a cell in the other filament. (C) The cells and their two haploid nuclei fuse to form a diploid **zygospore** (Z) and conjugation is complete. When conditions are favorable, the zygospore germinates by undergoing meiosis. Only one haploid nucleus survives and the zygospore cell develops into a new vegetative filament. Note the absence of spiral chloroplasts in the empty cells and in the zygospores.

had a chloroplast from primary endosymbiosis, the plastids derived from it are said to have been formed by **secondary endosymbiosis**.) In some modern species, the plastid is still present; in others, only red algal plastid DNA remains in the genome. More work needs to be done on this group before we have a clear picture.

- Alveolates may be either heterotrophic or autotrophic, but they all have small, membranous sacs beneath their cytoplasmic membrane. They are represented here by two ciliates—*Paramecium* (Fig. 3.20A) and *Stentor* (Fig. 3.20B). Ciliates are characterized by cilia covering their outer surface that provide motility when they sweep back-and-forth. Cilia also line the **oral groove** and sweep food particles inward toward the **cytostome** where it is engulfed.

  The alveolate pathogens *Balantidium coli* and *Plasmodium* are shown in Figures 12.33 and 12.34, and 12.35 through 12.38, respectively.

- The stramenopiles are characterized by a unique flagellum with three-parted lateral hairs emerging from it as well as some other physiological features. Even though diatoms (Fig. 3.21) lack flagella, they are so successful and are so frequently seen in aquatic samples that we have chosen them to represent the group. Diatoms are single-celled or colonial autotrophs. They have a distinctive golden-brown color due to the abundance of the pigment **fucoxanthin**. Their highly ornamented cell wall is made of silica embedded in an organic matrix and consists of two halves with one half overlapping the other in the same way the lid of a Petri dish overlaps its base. **Centric diatoms** have a round shape, whereas **pennate diatoms** are bilaterally symmetrical. Oil droplets are frequently visible.



3.20 SAR Supergroup (Alveolate Subgroup) ♦ (A) Shown is the ciliate *Paramecium bursaria*. Note the cilia (C) around the cell's edge and the prominent macronucleus (Ma) and micronucleus (Mi). Micronuclei are involved in genetic transfer between compatible mating types, whereas the macronucleus is responsible for governing cellular function. At the stage shown, the micronucleus is diploid and the macronucleus has multiple copies of the cell's genome. Cilia serve to move the cell as well as to sweep food into its cytostome ("mouth"). (B) Its trumpet shape, size (up to 2 mm), and beaded macronucleus (M) make *Stentor* an easy ciliate to identify. The white spot inside the dark coiled region is the cytostome. This species is naturally green; this is not a stained specimen.

3.21 SAR Supergroup (Stramenopile Subgroup) ♦ While diatoms lack the flagella characteristic of most stramenopiles, they are so ubiquitous we have chosen them to represent the group. Their cell wall is divided into two halves, one of which fits into the other like the lid and base of a Petri dish, and are composed of silica and organic material. Diatoms are divided into two groups based on their symmetry. Those with bilateral symmetry (A) are said to be pennate and those with radial symmetry (B) are said to be centric. Note the distinctive golden chloroplasts, the cell wall ornamentation, and the oil droplets (O).

Application

Familiarity with eukaryotic cells not only rounds out your microbiological experience, it is important to be able to differentiate eukaryotic from prokaryotic cells when examining environmental or clinical specimens.

In This Exercise

3

Most of this manual is devoted to prokaryotes, but in this exercise you will be given the opportunity to examine various eukaryotic microorganisms. This will not only serve to familiarize you with simple eukaryotes, but also give you practice using the microscope, measuring specimens, and making wet-mount preparations.

✓ Materials

Per Class

- ☐ Prepared slides or living cultures of a variety of protists and fungi (such as, *Amoeba, Saccharomyces, Euglena, Chlamydomonas, Volvox, Spirogyra, Paramecium, Stentor,* and diatoms.)

Per Student

- ☐ Clean glass slides and cover glasses
- ☐ Compound microscope with ocular micrometer
- ☐ Cytological stains (e.g., methylene blue, I_2KI)
- ☐ Methyl cellulose
- ☐ Immersion oil
- ☐ Cotton swabs
- ☐ Lens paper
- ☐ Lens cleaning solution

Procedure

1. Obtain a microscope and place it on the table or workspace. Check to be sure the stage is all the way down and the scanning objective is in place.
2. Begin with a prepared slide. Clean it with a tissue if it is dirty, and then place it on the microscope stage. Center the specimen under the scanning objective.
3. Follow the instructions given in Exercise 3-1, to bring the specimen into focus at the highest magnification that allows you to see the entire structure you want to view.
4. Practice scanning with the mechanical stage until you are satisfied that you have seen everything interesting to see. Sketch what you see in the table provided on the data sheet, page 167.
5. Measure cellular dimensions and record these in the table provided on the data sheet.
6. Repeat with as many slides as you have time for.
7. Prepare wet mounts of available specimens by following the procedural diagram in Figure 3.14. Methyl cellulose may be added to the wet mount if you have fast swimmers. Sketch what you see and record cellular dimensions in the chart provided on the data sheet.
8. When you are finished observing specimens, blot the oil from the oil-immersion lens (if used) with a lens paper and do a final cleaning with a cotton swab and alcohol or lens cleaning solution. Dry the lens with a clean swab.
9. Return all lenses and adjustments to their storage positions before putting the microscope away.

References

Reece, Jane B., Lisa A. Urry, Michael L. Cain, Steven A. Wasserman, Peter V. Minorsky, and Robert B. Jackson. Chap. 28 in *Campbell Biology* 10th ed. Boston: Pearson, 2014.

Madigan, Michael T., John M. Martinko, David Stahl, and David P. Clark. Chap. 12 in *Brock's Biology of Microorganisms,* 13th ed. San Francisco: Pearson/Benjamin Cummings, 2011.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-3

Microscopic Examination of Eukaryotic Microbes

OBSERVATIONS AND INTERPRETATIONS

1 Fill in the table for each eukaryotic microbe you observe.

Organism (include wet mount or prepared slide)	**Sketch** (include magnification and stain, if any)	**Dimensions**	**Identifying Characteristics** (list only those you observed)

Organism (include wet mount or prepared slide)	**Sketch** (include magnification and stain, if any)	**Dimensions**	**Identifying Characteristics** (list only those you observed)

3

QUESTIONS

1 *What features did the cells you observed have in common? How were they different?*

2 *If you observed the same organism on a prepared slide and a wet mount, how did the images compare?*

Microscopic Examination of Freshwater Microbes

EXERCISE 3-4

Theory

At some time in your life, you undoubtedly have passed a lake or river and casually noticed that a green goo was growing on the water's surface or that the mud was dark in color. Perhaps you were put off by a foul smell. Microorganisms cause all of these—and more. Today you will see the previously unseen microbes that caught your attention.

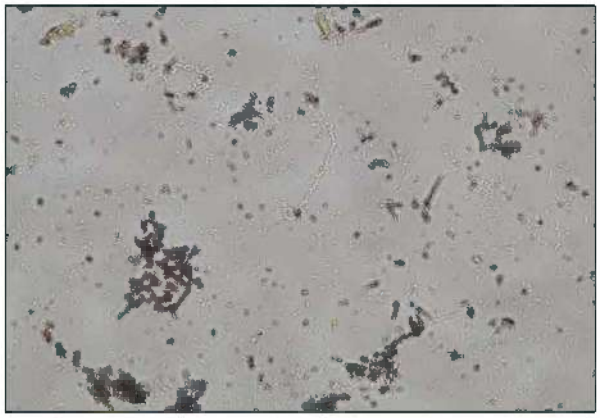
Bacteria are the smallest organisms that you will see in your preparation (Fig. 3.22). Without staining they will appear as small (about 10 µm or less), transparent cells in the shapes of rods (bacilli), spheres (cocci), and spirals (spirilla). Sometimes they will be joined together in clusters or chains. Watch for swimming bacteria. Follow their path and see if they swim in a straight line or move along an irregular course.

Cyanobacteria are in the Domain *Bacteria*. They are easily seen without staining because of their combination of photosynthetic pigments, which confer on them a bluish-green color. In fact, they were formerly known as "blue-green algae." When they are single-celled, they are about the size of bacteria. But when they are found in chains, called **trichomes**, they are easily visible at high-dry magnification. Some trichomes have an extracellular sheath. Also, watch for slow, gliding motility of the trichomes. Trichomes often have specialized cells, including **heterocysts**, which are nitrogen-fixing cells, and **akinetes**, which are resistant spores (Figs. 3.23 to 3.31).

Eukaryotes are a heterogeneous group and are divided into four supergroups: Archaeplastida, SAR, Excavata, and Unikonta, and they all have microscopic representatives. In fact, they are dominated by microscopic species. These are described in the Theory section of Lab Exercises 3-3, 12-1, and 12-3.

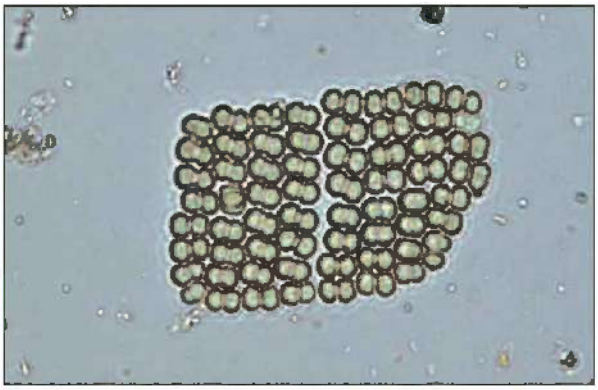
Following is a brief list of what you might observe in your freshwater samples and some key features that will help you identify them.

3.23 ***Anabaena*** ♦ The trichomes of this gliding cyanobacterium may possess thick-walled spores called **akinetes** (A) and specialized, nitrogen-fixing cells called **heterocysts** (H). Trichomes are of variable lengths but are approximately 20 µm in width.

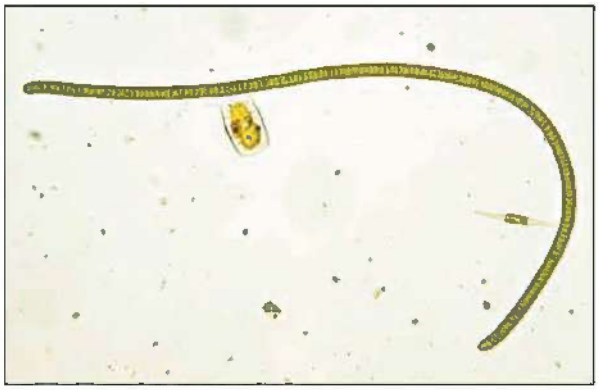


3.22 **Bacteria** ♦ Bacterial cells (the small, light objects) are shaped like spheres, rods, or spirals, and all are represented in this micrograph. These are the smallest cells you will see. Most are in the range between 1 µm and 10 µm.

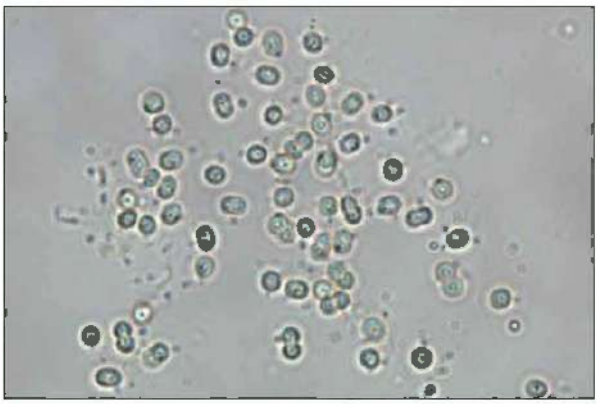
3.24 ***Lyngbya*** ♦ *Lyngbya* trichomes are distinguished from *Oscillatoria* (Fig. 3.28) by the distinctive sheath (S). The trichomes are approximately 20 µm in width.



3.25 ***Merismopedia*** ♦ Cell division in two perpendicular directions produces the planar arrangement characteristic of this genus. The cells are enclosed in a mucilaginous sheath and are approximately 1 µm–2 µm in diameter.



3.28 ***Oscillatoria*** ♦ Trichomes are formed from disk-shaped cells that are approximately 10 µm in width. Watch for gliding motility.



3.26 ***Microcystis*** ♦ Mucilage surrounds colonies of this planktonic genus. The dark spots are gas "pseudovacuoles" that allow it to float in the water. Some species produce an animal toxin. Cells are approximately 3 µm in diameter.

3.29 ***Spirulina*** ♦ *Spirulina* is perhaps the most distinctive cyanobacterium because of its helical shape. This genus is sold in health food stores as a dietary supplement.

3.27 ***Nostoc*** ♦ This genus is easily identified macroscopically because of its globular colonies and thick, rubbery mucilage. Trichomes are composed of spherical cells and form a tangled mass within the colony. Note the terminal spherical heterocysts. Trichomes are about 5 µm in width.

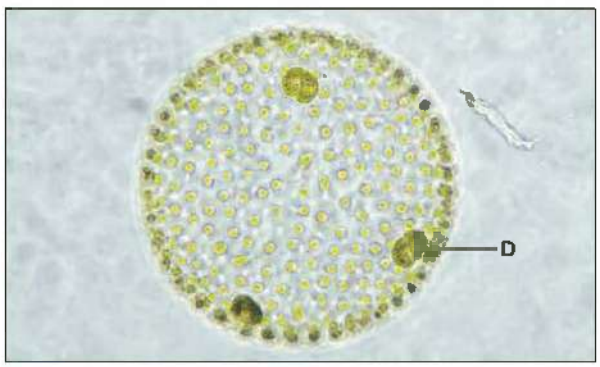
3.30 ***Rivularia*** ♦ Macroscopically, trichomes form globular, gelatinous masses. Microscopically, they are tapered to a fine point. Note the round, basal heterocysts. Akinetes are absent.

3.31 *Chroococcus* ♦ The sheathed cells of this genus occur as singles, pairs, or tetrads, with the latter two being more hemispherical than spherical.

Archaeplastida (Chlorophytes and Charophytes)

- Chlorophytes are green algae. They will be seen as single cells, colonies, and branched or unbranched filaments (Figs. 3.32 to 3.39). You may also see sheets of cells. Look for green chloroplasts, thick cell walls, vacuoles, and nuclei. Many also exhibit pyrenoids in the chloroplasts where photosynthetic products are stored.

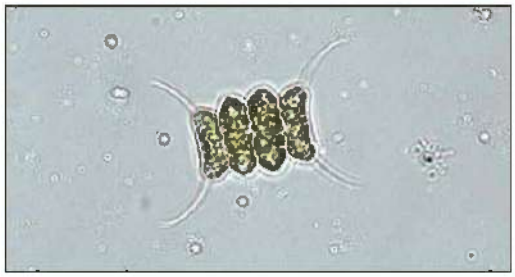
3.32 *Chlamydomonas* ♦ This unicellular green alga has two flagella (F) and a single, cup-shaped chloroplast. A red-orange stigma (S) is found within the chloroplast and is involved in phototaxis. Cells are approximately 30 µm long and 20 µm wide.



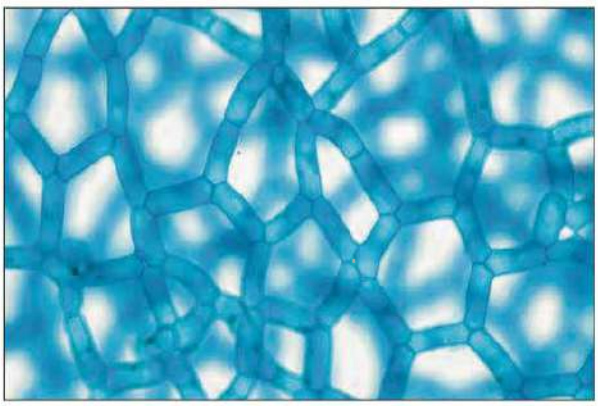
3.33 *Volvox* ♦ This is a colonial green alga made of cells similar in shape to *Chlamydomonas*. Daughter colonies (D) form asexually from special cells in the parent colony. Release of the daughter colony involves its eversion as it exits through an enzymatically produced pore. Mature colonies can reach a size visible to the naked eye.

3.34 *Oedogonium* ♦ (A) *Oedogonium* is a large chlorophyte genus composed of unbranched filaments. Division of cells within the filament results in its elongation and produces distinctive division scars (S). (B) The cylindrical cells of this filament are interrupted by two eggs, also called "oogonia" (O).

3.35 *Ulothrix* ♦ *Ulothrix* is another unbranched, filamentous chlorophyte. Its chloroplasts are found near the cell wall and form either a complete ring or an incomplete ring around the cytoplasm. Prominent pyrenoids (yellowish dots) are usually present.



3.36 *Scenedesmus* ♦ This common chlorophyte consists of 4 or 8 cells joined along their edges. The cells on each end have distinctive spines. Asexual reproduction occurs as each cell of the colony divides to produce a new colony within the confines of its cell wall. These are subsequently released as the parental cell wall breaks down. Cells range from 30 µm to 40 µm across.



3.37 *Hydrodictyon* ♦ The cells of this green alga form open connections with 4 (sometimes more) of its neighbors to produce a complex network of multinucleate cells. This explains its common name: the "water net." Cells may reach 1 cm in length, and each contains a single chloroplast with numerous pyrenoids. This specimen is from a culture.

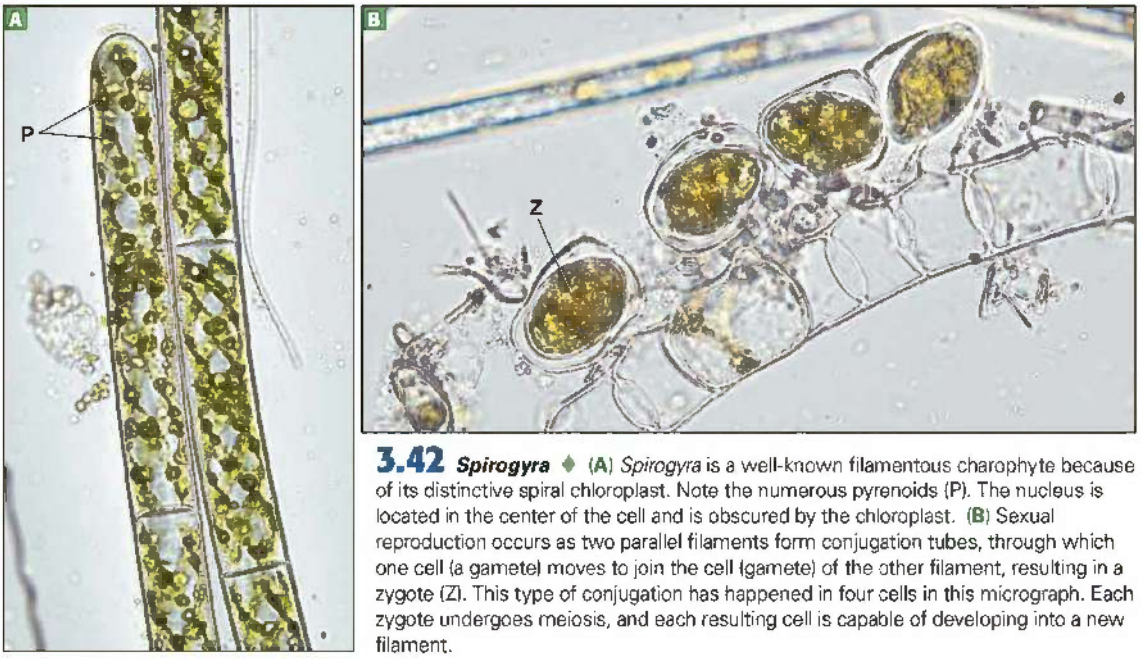
3.38 *Pediastrum* ♦ The colonies of *Pediastrum* are angular and starlike, and the surface cells often contain bristles that are thought to be used for buoyancy. The cell number and arrangement are constant for each species. Each cell has the potential to produce a colony of the same number and arrangement of cells as the parent colony.

- The distinctions between charophytes (Figs. 3.40 to 3.42) and chlorophytes are complex and often not visible to the light microscopist. Two groups are pretty easy to identify, however. These are the desmids, composed of paired "semicells," and species of the genus *Spirogyra* with their spiral chloroplasts.

3.39 *Cladophora* ♦ If the specimen is green and made of branched filaments with elongated cells, it is most likely a species of *Cladophora*. The cells are multinucleate and the single chloroplast has numerous pyrenoids that may look like a network of unconnected pieces. This high magnification shows a single branch as well as numerous diatoms growing on the filaments as epiphytes.

3.40 *Cosmarium* ♦ Desmids, such as *Cosmarium*, are characterized by paired "semicells." In this species of *Cosmarium* (one of more than 1,000!) two chloroplasts—one at each end—are present in each cell. They are charophytes.

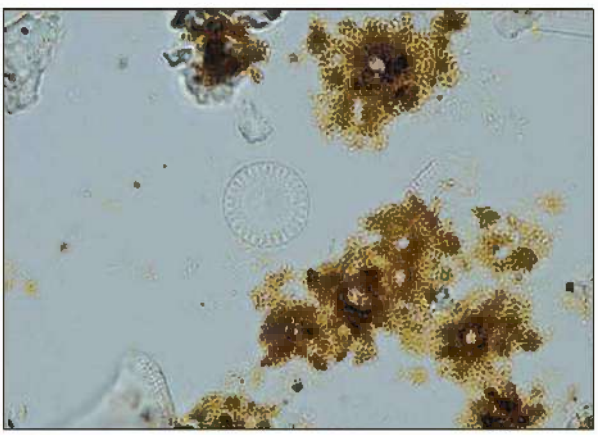
3.41 *Closterium* ♦ *Closterium* is another desmid composed of two cells, but lacking the distinct constriction typical of most desmids. Vacuoles are present at the outer ends of the cells.



3.42 ***Spirogyra*** ♦ (A) *Spirogyra* is a well-known filamentous charophyte because of its distinctive spiral chloroplast. Note the numerous pyrenoids (P). The nucleus is located in the center of the cell and is obscured by the chloroplast. (B) Sexual reproduction occurs as two parallel filaments form conjugation tubes, through which one cell (a gamete) moves to join the cell (gamete) of the other filament, resulting in a zygote (Z). This type of conjugation has happened in four cells in this micrograph. Each zygote undergoes meiosis, and each resulting cell is capable of developing into a new filament.

SAR Supergroup (Stramenopiles, Alveolates, and Rhizarians)

Diatoms (Figs. 3.43 to 3.52) are frequently abundant representatives of the stramenopile group. They are photosynthetic and have a distinctive golden-brown color from the pigment **fucoxanthin** located in the **chromoplasts**, which may be of variable shape. Oil droplets are also frequently visible. Cell shapes are either round (**centric**) or bilaterally symmetrical and elongated (**pennate**). The cell wall is made up of silica embedded in an organic matrix and consists of two halves with one half overlapping the other in the same way the lid of a Petri dish overlaps its base. The combination of both halves is called a **frustule**. (Often, diatoms can be identified from the frustules of deceased cells.) Some pennate diatoms are motile and have a central, longitudinal line called a **raphe**. Look for these creeping diatoms!

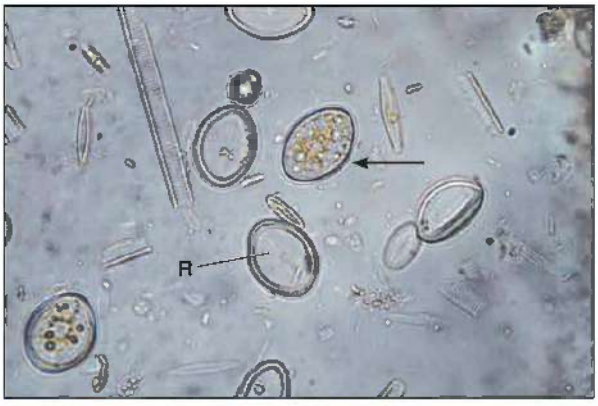


3.43 ***Cyclotella*** ♦ In this micrograph we are seeing the empty frustule of *Cyclotella*, a centric diatom. The valve has a peripheral zone of radiate (like spokes of a wheel) ridges.

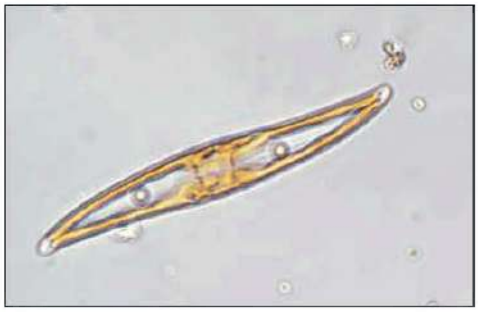
3.44 ***Tabellaria*** ♦ This distinctive diatom forms zigzag colonies. Note the golden-brown chloroplasts.

3.45 ***Cymbella*** ♦ This *Cymbella* species has bilaterally asymmetric valves and an H-shaped chloroplast. Note the prominent oil droplets.

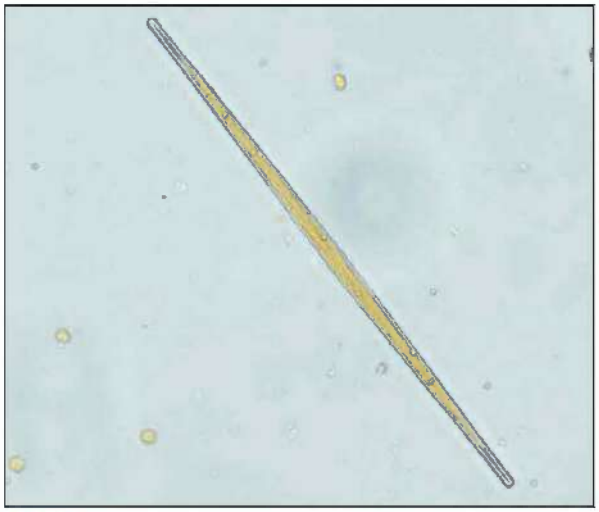
3.46 ***Bacillaria*, also known as *Nitzschia*** ♦ Members of this genus glide with individual cells sliding over one another. In this colony, the top cells are in the process of gliding to the right over the lower cells.



3.47 ***Cocconeis*** ♦ These elliptical diatoms have transverse lines and a thick marginal band. There is also a faint longitudinal line called a raphe (R). Several empty frustules are visible, as is one living specimen (arrow).

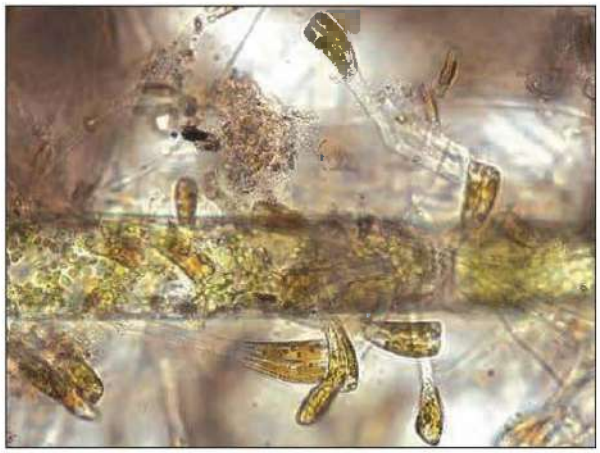


3.48 ***Gyrosigma*** ♦ All species of this genus have sigmoid-shaped cells. Note the golden-brown chloroplasts at the edge and two prominent oil droplets.



3.49 ***Synedra*** ♦ These diatoms are distinctive because of their long, needle-shaped cells. They occur singly or sometimes in groups of cells attached at their ends, radiating outward like spokes of a wheel.

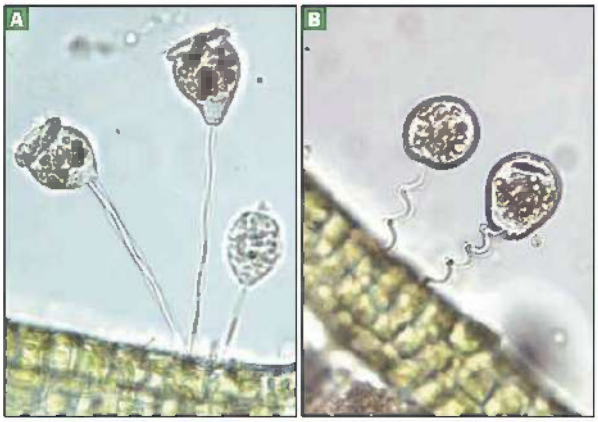
3.50 ***Fragillaria*** ♦ These rectangular cells form ribbons with cells attached side-by-side. Note the golden-brown chloroplast and oil droplets.



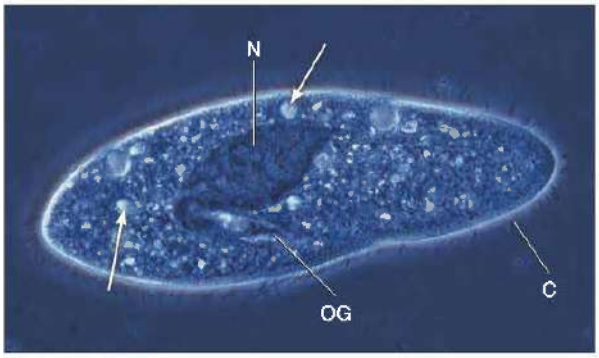
3.51 *Gomphonema* ♦ Wedge-shaped cells on branched stalks characterize the species of *Gomphonema*.

3.52 *Navicula* ♦ All of the many species of *Navicula* are shaped like a cigar or a boat. Cells are identifiable by prominent transverse lines converging on the central open space. A prominent longitudinal line is also present.

Many alveolates possess cilia for locomotion and feeding and are informally referred to as "ciliates" (Figs. 3.53 to 3.55). Some are free swimming while others are attached to a surface. Look for an oral groove, contractile vacuoles that pump water out of the cell, and food vacuoles.



3.53 *Vorticella* ♦ (A) *Vorticella* is a genus of stalked, inverted bell-shaped ciliates. A crown of cilia surround the oral groove and are involved in feeding. (B) *Vorticella* is capable of retraction when the stalk coils. If you find a *Vorticella* spend some time watching it until it recoils. It is worth the wait (which shouldn't be more than a minute).



3.54 *Paramecium* (Phase contrast) ♦ *Paramecium* may be one of the most famous ciliated unicellular eukaryotes. Notice the cilia (C), the oral groove (OG), and nucleus (N). The white circles (arrows) are food vacuoles.

3.55 *Trachelocerca* ♦ This ciliate has the general appearance of *Paramecium* but is usually more elongated and has a prominent contractile vacuole (V) used for pumping water out of the cell in the posterior.

Dinoflagellates (Fig. 3.56) are also alveolates and are typically unicellular and autotrophic. Most have two flagella: one protruding from the cell and the other positioned in a groove encircling the cell.

3.56 ***Ceratium*** ♦ Dinoflagellates have two flagella. One flagellum is found in a groove (girdle) (G) encircling the cell, whereas the other is elongated and is oriented posteriorly. This species has one anterior horn and three posterior horns and is covered with cellulose plates.

Heliozoans (Fig. 3.57) are spherical, unicellular, phagocytic cells characterized by projections called **axopodia**, used in feeding. Their sunburst appearance accounts for their name. They are classified as rhizarians.

3.57 ***Heliozoan*** ♦ These spherical, sun-shaped (hence, *helio*) organisms are among the most beautiful unicellular eukaryotes. They are spherical and have thin extensions called **axopodia**, which are used in feeding. They are not rigid but, rather, are extensions of ectoplasm and can be bent or retracted.

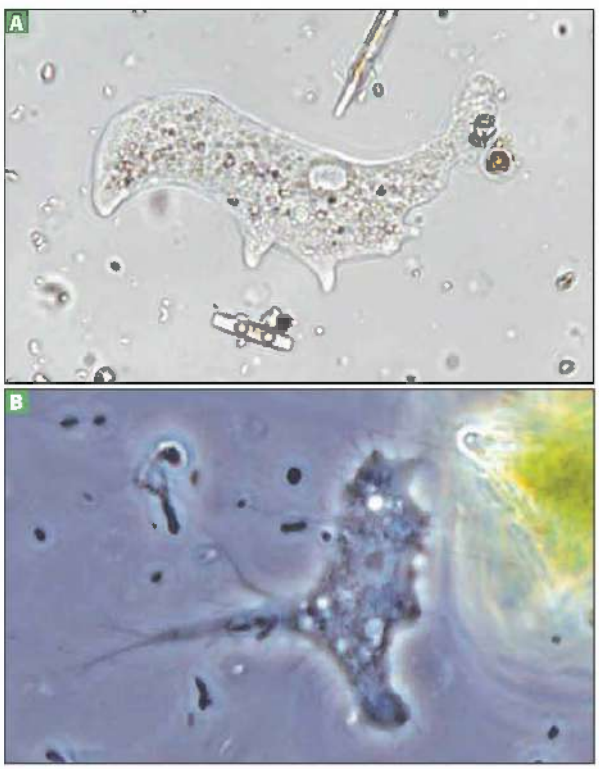
Excavata (Euglenozoa)

Euglenozoans (Fig. 3.58) are unicellular mixotrophs. They (usually) have green chloroplasts and photosynthesize when light is available, but they are capable of heterotrophy when light is not available. Look for chloroplasts and one or two flagella that emerge from an invagination of the anterior (forward) cytoplasmic membrane. A red photoreceptor called an **eyespot** at the cell's anterior is another distinctive feature.

3.58 ***Euglena*** ♦ *Euglena* is a large genus of mixotrophic flagellates. Most species have chloroplasts, which are discoid in this specimen. A red "eyespot" (E) is located in the colorless anterior of the cell. The anterior and posterior flagella (F) are visible on the lower individual, and the flexibility of euglenids is demonstrated by the other. The brown splotches to the right are out-of-focus cells.

Unikonta (Amoebozoa and Animalia)

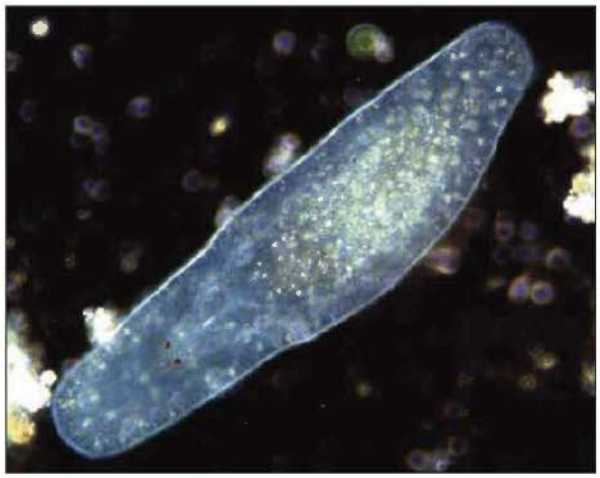
Amoebas (Figs. 3.59 and 3.60) move by producing temporary, lobe-shaped **pseudopodia** that also serve as feeding structures. In this latter role, they form and subsequently engulf their microorganismal food. Look for pseudopods, food vacuoles, and the nucleus.



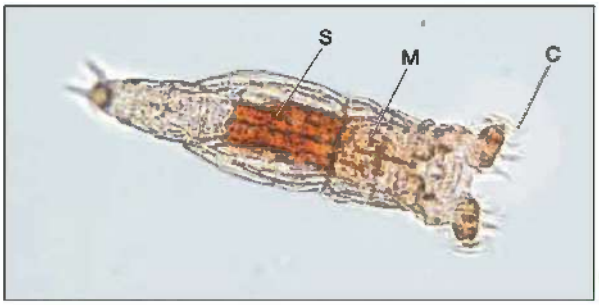
3.59 ***Amoeba*** ♦ Cells with flowing cytoplasm and a constantly changing shape are likely to be amoebas. They move and capture prey by extending pseudopods ("false feet") outward. (**A**) This bright-field micrograph clearly illustrates the difference between the cytoplasm at the periphery of the cell (ectoplasm) with the cytoplasm in the interior (endoplasm). The nucleus is visible, as are numerous food vacuoles seen as golden spots—probably an indication of this individual's food preferences! (**B**) This is a totally different amoeba, interesting for its complex pseudopods.

3.60 ***Astramoeba*** ♦ This amoeba possesses long, thin pseudopods that may coil when disturbed. Movement is slow, but the irregular shape tends to remain unchanged for long periods of time. These cells are less than 2 mm across (including pseudopods).

Invertebrate animals (Figs. 3.61 to 3.66) include all animal phyla that are not in the phylum Chordata—in other words, the majority of animals—and some will inevitably make an appearance in your wet-mount preparation. A few examples are discussed in the photograph captions.



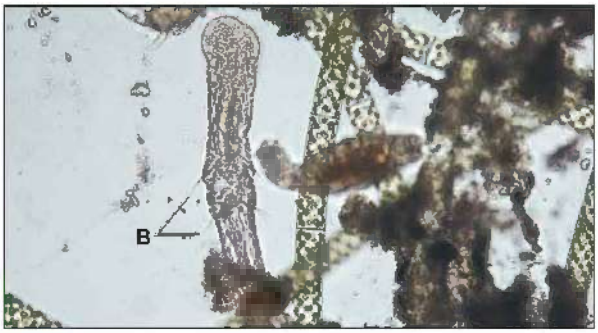
3.61 **Planarian** ♦ This flatworm has a simple nervous system and an incomplete digestive tract. Cilia on its ventral surface allow it to move. The structures that look like eyes are, in fact, photoreceptors but they don't produce an image. They just detect light intensities.



3.62 **Rotifer** ♦ These are commonly called "wheel animals," and it takes only a quick glance to see why. At their "head" is a crown, or **corona**, of cilia (C), which sweep food particles into their mouth and also provide a means of locomotion. Perhaps the most prominent part of the cylindrical body is the **mastax** (M), which is a grinding organ just posterior to the mouth. You will see it move. In some rotifers it can be extended to capture prey. In this unstained specimen, the stomach (S) is also easily seen. Also look for the spurs extending from the posterior, which are used for attachment.

3.63 **Gastrotrich** ♦ Gastrotrichs have a complete digestive tract (the darker, central region in this specimen), a simple nervous system and sensory organs concentrated in their "head" region.

3.64 **Nematode Worm** ♦ Nematodes are also known as roundworms and they are abundant in all habitats. They are unsegmented and have a complete digestive tract; that is, they have a mouth (M) and an anus (A). The most notable feature of these worms is how they thrash about. You may see them directly, or your eyes may be drawn to plant debris and soil particles being stirred up. Look more closely and you will probably find a nematode!



3.65 Annelid Worm ♦ The bodies of annelid worms are divided into repeating segments, and they also have a complete digestive tract. This is a micrograph of a polychaete worm and is easily identified by the presence of bristles (B) on each segment.

3.66 Arthropod ♦ Arthropods, the largest animal phylum, are characterized by their jointed appendages. They comprise such diverse animals as barnacles, spiders, crabs, insects, and many others. Shown here is a crustacean, probably of the ostracod group, which is closely related to the "water fleas," such as *Daphnia*.

Application

This lab is designed to have you practice using the microscope and experience the awe-inspiring microbial diversity and beauty of microbial life found in natural water sources.

In This Exercise

You will observe pond, river, or other source of freshwater life and identify representative microorganisms from diverse biological taxa such as *Bacteria*, cyanobacteria, green algae, protists, and a variety of invertebrate animals. Although invertebrates are not considered to be microorganisms, you will see them, so they are covered here.

✓ Materials

Per Student Group

- ☐ Pond water—the goopier the better! Mud and green stuff is good!
- ☐ Microscope slides
- ☐ Cover slips
- ☐ Compound microscope with ocular micrometer
- ☐ Plastic transfer pipettes
- ☐ One dropper bottle of 1.5% w/v methylcellulose

Procedure

1. Obtain a clean glass microscope slide and cover slip.
2. Prepare a wet-mount slide by following the procedural diagram (Fig. 3.14). With a transfer pipette, place a drop of pond water on the slide. Take your sample from a region with some goo or mud. You may see some organisms if you take water from the surface, but you will see more where there is organic material.
3. Carefully place a cover slip over the drop. Try not to trap any air underneath it, but this may be impossible if you have sand grains or other large objects in the drop. If you do, take off the cover slip and try to remove the offending objects. It may take a new drop of water before you replace the cover slip. In some cases, it is just easier to wipe off the slide, start over, and avoid the sand.
4. Examine the water drop using the scanning, low, and high-dry objectives.
5. If the swimmers are moving too quickly to observe, remove the cover slip and add a drop of methyl cellulose to slow them. Replace the cover slip (avoiding air bubbles) and continue your observations. Be careful not to get the methyl cellulose on any objective lens or the stage. If you do, clean it up immediately with lens paper for the lenses and tissue for the stage.
6. Use Figures 3.22 to 3.66 to place the organisms you observe into the taxa described in the discussion of Theory.
7. Record your observations and answer the questions on the data sheet, page 179.

References

Bold, Harold C. and Michael J. Wynne. *Introduction to the Algae*, 2nd ed. Englewood Cliffs, NJ: Prentice-Hall, 1985.

Graham, Linda E., James M. Graham, and Lee W. Wilcox. *Algae*, 2nd ed. San Francisco: Pearson Benjamin Cummings, 2009.

Jahn, Theodore Louis, Eugene C. Bovee, Francis Floed Jahn, John Bamrick, Edward T. Cauley, and Wm G. Jaques. *How to Know the Protozoa*, 2nd ed. Columbus, OH: McGraw-Hill Companies, 1978.

Lee, Robert Edward. *Phycology*, 4th ed. New York: Cambridge University Press, 2008.

Needham, James G. and Paul R. Needham. *A Guide to the Study of Fresh-water Biology*, 5th ed. San Francisco: Holden-Day, 1962.

Prescott, G., John Bamrick, Edward T. Cauley, and Wm G. Jaques. *How to Know the Protozoa*, 3rd ed. Columbus, OH: McGraw-Hill Companies, 1978.

Vinyard, William C. *Diatoms of North America*. Eureka, CA: Mad River Press, 1979.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-4

Microscopic Examination of Freshwater Microbes

OBSERVATIONS

1 Carefully draw and label as much structural detail as you can of four representative organisms ***you actually observed.*** Draw only a single organism in each circle, and take pride in your artwork!

Organism _____

Magnification _____

Dimensions _____

Organism _____

Magnification _____

Dimensions _____

Organism _____

Magnification _____

Dimensions _____

Organism _____

Magnification _____

Dimensions _____

QUESTIONS

1 *For each of the four organisms you identified, list the characteristics you used in making your identification.*

a. *Organism:* _____

Characteristics: _____

b. *Organism:* _____

Characteristics: _____

c. *Organism:* _____

Characteristics: _____

d. *Organism:* _____

Characteristics: _____

Bacterial Structure and Simple Stains

Importance of Contrast

In Exercise 3-1 you were introduced to two of the three important features of a microscope and microscopy: magnification and resolution. A third feature is **contrast**. To be visible, the specimen must contrast with the background of the microscope field. Because cytoplasm is essentially transparent, viewing cells with the standard light microscope is difficult without stains to provide that contrast. In the upcoming exercises, you will learn how to correctly prepare a bacterial smear for staining and how to perform simple and negative stains. Details of cell structure (cell morphology, size, and arrangement) then may be seen more easily. In a medical laboratory these usually are determined with a Gram stain (Exercise 3-7), but you will be using simple stains as an introduction to the staining process, as well as an introduction to these cellular characteristics.

Bacterial Cell Morphologies

Bacterial cells are much smaller than eukaryotic cells (Fig. 3.12) and come in a variety of **morphologies** (shapes) and **arrangements**. Determining cell morphology is an important first step in identifying a bacterial species. Cells may be spheres (**cocci**, singular **coccus**), rods (**bacilli**, singular **bacillus**) or spirals (**spirilla**, singular **spirillum**). Variations of these shapes include slightly curved rods (**vibrios**), short rods (**coccobacilli**) and flexible spirals (**spirochetes**). Examples of cell morphologies are shown in Figures 3.67 through 3.74. In Figure 3.74, *Corynebacterium xerosis* illustrates pleomorphism. A variety of cell shapes—slender, ellipsoidal, or ovoid rods—may be seen in a given sample.

Bacterial Cell Arrangements

Cell arrangement, determined by the number of planes in which division occurs and whether the cells separate after division, is also useful in identifying bacteria (Table 3-4). Spirilla rarely are seen as anything other than single cells, but cocci and bacilli do form multicellular associations. Because cocci exhibit the most variety in arrangements, they are used for illustration in Figure 3.75. If the two daughter cells remain attached after a coccus divides, a **diplococcus** is formed. The same process happens in bacilli that produce **diplobacilli**. If the cells continue to divide in the same plane and remain attached, they exhibit a **streptococcus** or **streptobacillus** arrangement.

TABLE **3-4** **Cell Arrangements Found Associated with Different Cell Morphologies**

Cell Arrangement	Cell Morphologies with the Cell Arrangement
Single cells	Cocci, bacilli, spirilla, spirochaetes, and vibrios
Pairs of cells ("diplo–")	Cocci and bacilli
Chains of cells ("strepto–")	Cocci and bacilli
Tetrads	Cocci
Cube	Cocci
Irregular cluster ("staphylo–")	Cocci
Palisade and angular	Bacilli

If a second division occurs in a plane perpendicular to the first, a **tetrad** is formed. A third division plane perpendicular to the other two produces a cube-shaped arrangement of eight cells called a **sarcina**. Tetrads and sarcinae are seen only in cocci. If the division planes of a coccus are irregular, a cluster of cells is produced to form a **staphylococcus**. Figures 3.76 through 3.81 illustrate common cell arrangements.

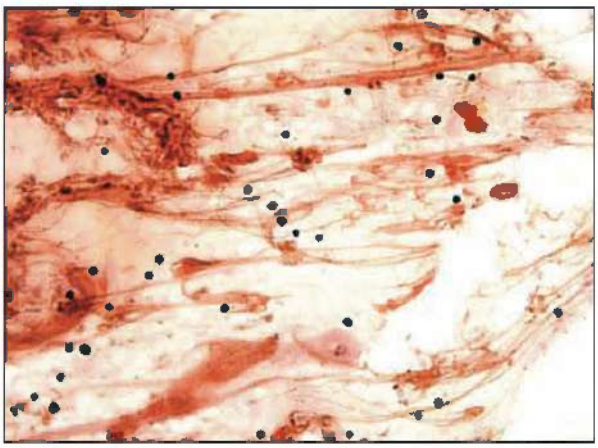
Arrangement and morphology often are easier to see when the organisms are grown in a broth rather than a solid medium, or are observed from a direct smear. If you have difficulty identifying cell morphology or arrangement, consider transferring the organism to a broth culture and trying again.

One last bit of advice: Do not expect nature to conform perfectly to our categories of morphology and cell arrangement. These are convenient descriptive categories that will not be applied easily in all cases. When examining a slide, look for the most common morphology and most complex arrangement. Do not be afraid to report what you see. For instance, it is okay to say, "Cocci in singles, pairs, and chains."

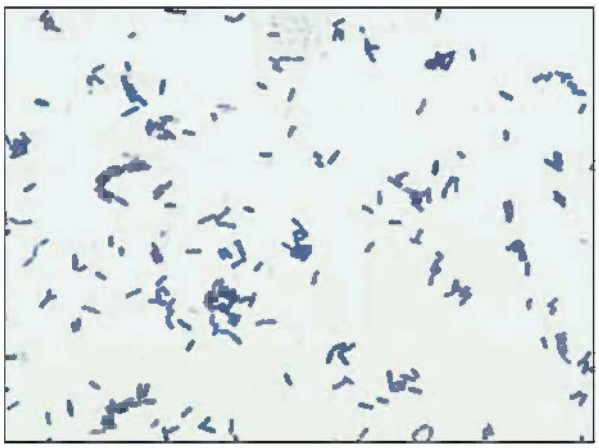
Surface Area and Volume of Cells

Cells are three-dimensional objects with a surface that contacts the environment and a volume made up of cytoplasm. The ability to transport nutrients into the cell and wastes out of the cell is proportional to the amount of surface area doing the transport. The demand for nutrients and production of wastes is proportional to a cell's volume. It is a mathematical fact of life that as an object gets bigger, its volume increases more rapidly than its surface area. Therefore, a cell can achieve a size at which its surface area is not adequate to supply the nutrient needs of its cytoplasm. That is, its **surface-to-volume** ratio is too small. At this point, a cell usually divides its volume to increase its surface area. This phenomenon is a major factor in limiting cell size and determining a cell's habitat.

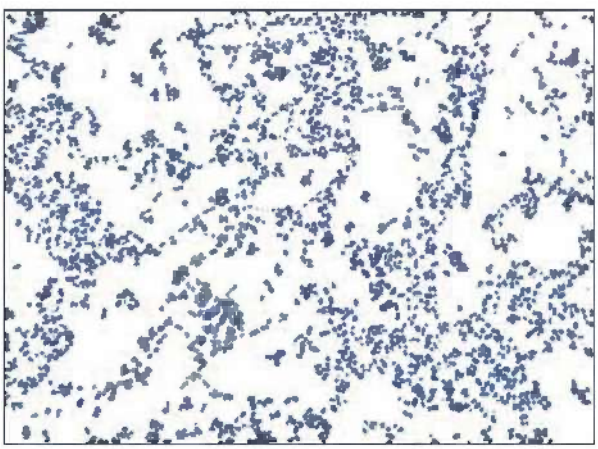
Bacilli, cocci, and spirilla with the same volume have different amounts of surface area. A sphere has a lower surface to volume ratio than a bacillus or spirillum of the same volume. A streptococcus, however, would have approximately the same surface to volume ratio as a bacillus of the same volume. ♦



3.67 Single Cocci from a Nasal Swab (Gram Stain) ♦ This direct smear of a nasal swab illustrates unidentified cocci (dark circles) stained with crystal violet. Each cell is about 1 µm in diameter. The red-orange background material is mostly mucus.

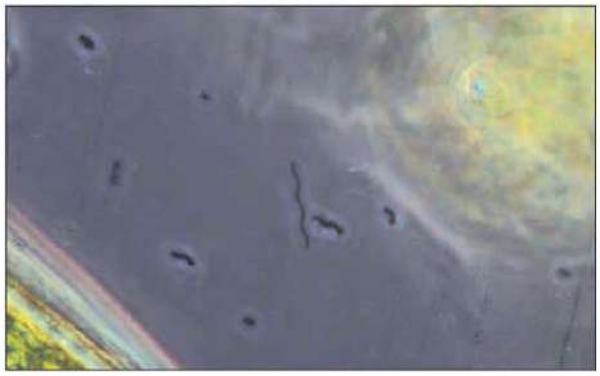


3.69 "Typical" *Bacillus* (Crystal Violet Stain) ♦ Notice the variability in rod length (because of different ages of the cells) in this stain of the soil organism *Bacillus subtilis* grown in culture. Cell dimensions are 2 µm–3 µm long by 0.7 µm wide.



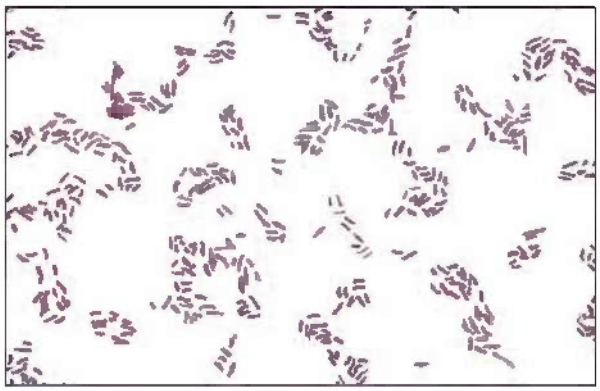
3.68 Ovoid Coccus (Gram Stain) ♦ *Lactococcus lactis* is an elongated coccus that a beginning microbiologist might confuse with a rod. Notice the slight elongation of the cells, and also that most cells are not more than twice as long as they are wide. *L. lactis* is found naturally in raw milk and milk products, but these cells were grown in culture. Cell dimensions are about 1 µm–2 µm long by 1 µm wide.

3.70 Long, Thin *Bacillus* (Gram Stain) ♦ The cells of *Aeromonas sobria*, a freshwater organism, are considerably longer than they are wide. These cells were grown in culture and their dimensions are 1 µm–3.5 µm long by 0.5 µm wide. Notice that a cell can be a bacillus without being in the genus *Bacillus*.

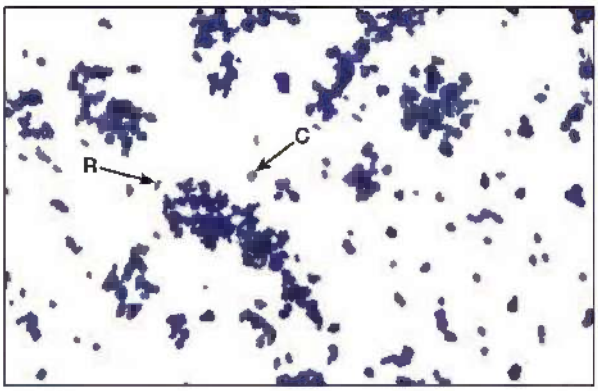


3.71 Two Different Spirilla (Phase Contrast Wet Mount) ♦ The two different spirilla are undoubtedly different species based on their different morphologies: one is long and slender with loose spirals, the other is shorter and fatter with tighter coils. Cell dimensions are less than 1 µm wide and up to 35 µm long.

3.72 Spirochete (Bright-Field Wet Mount) ♦ Spirochaetes are tightly coiled, flexible rods. This bright-field micrograph shows a marine species of *Spirochaeta* (arrow) Notice the bend in the center of the cell. Cell dimensions of the genus are 5 µm–500 µm long (!) by less than 1 µm wide.

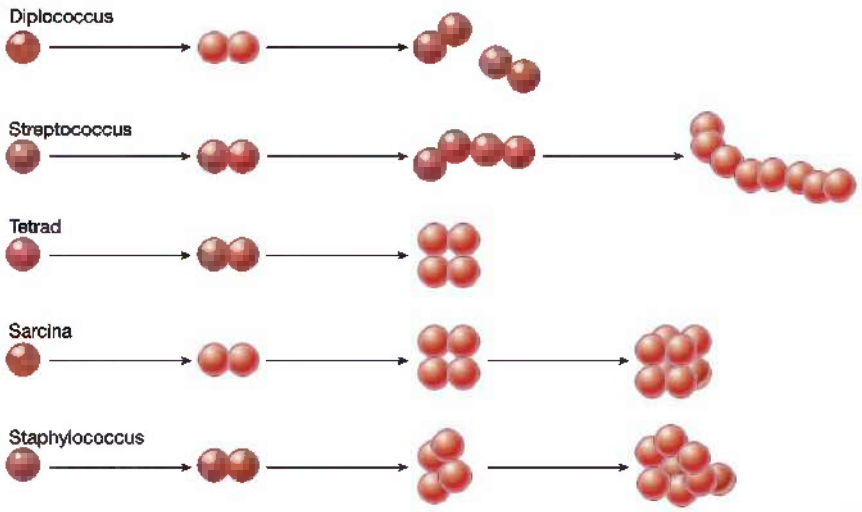


3.73 Marine *Vibrio* (Gram Stain) ♦ Slightly curved rods are demonstrated by this unidentified species isolated from a Southern California estuary. Notice that not all rods are curved. Cells are approximately 2 µm–3 µm long by 0.5 µm wide.

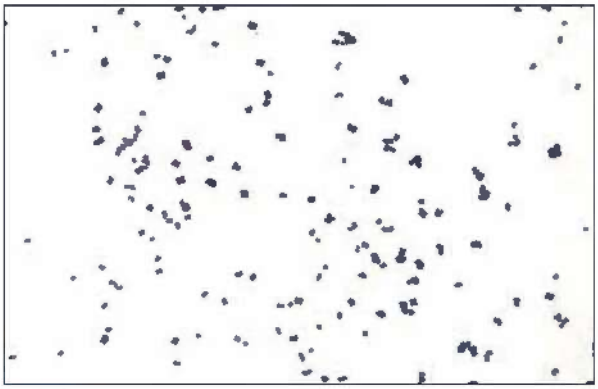


3.74 Bacterial Pleomorphism (Gram Stain) ♦ Some bacterial species grow in a variety of shapes and are said to be pleomorphic. Notice that in this specimen the rods of *Corynebacterium xerosis* range from plump cocci (C) to short, slender rods (R). *C. xerosis* is a normal inhabitant of skin and mucous membranes and is generally not considered to be pathogenic.

3.75 Division Patterns Among Cocci ♦ Diplococci have a single division plane and the cells generally occur in pairs. Streptococci also have a single division plane, but the cells remain attached to form chains of variable length. If there are two perpendicular division planes, the cells form tetrads. Sarcinae have divided in three perpendicular planes to produce a regular cuboidal arrangement of cells. Staphylococci have divided in more than three planes to produce a characteristic grapelike cluster of cells. (***Note:*** Rarely will a sample be composed of just one arrangement. Report what you see, and emphasize the most complex arrangement.)

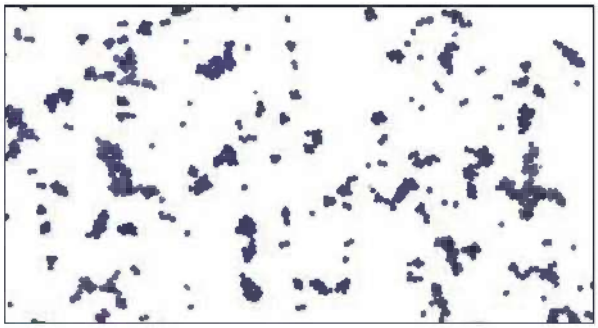


3.76 Diplococcus Arrangement in Nasal Mucous (Gram Stain) ♦ Most cells are in pairs, but a few are singles because they have yet to divide. Expect to see a mixture of cell arrangements representing stages before the "final" one.

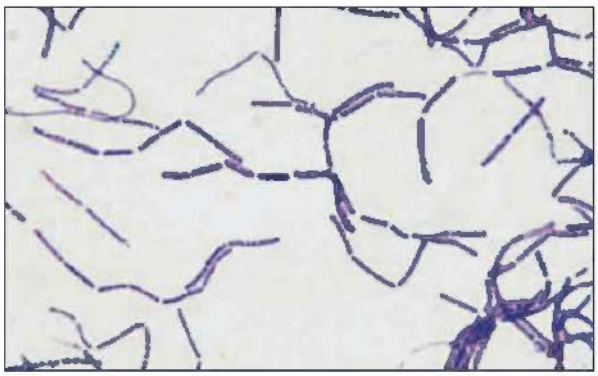


3.77 Tetrad Arrangement (Gram Stain) ♦ *Micrococcus luteus* grows in squared packets of cells, evident even when they are bunched together. This slide shows cells grown in culture, but *M. luteus* are normal inhabitants of mammalian skin and soil. Each cell is approximately 1 µm in diameter.

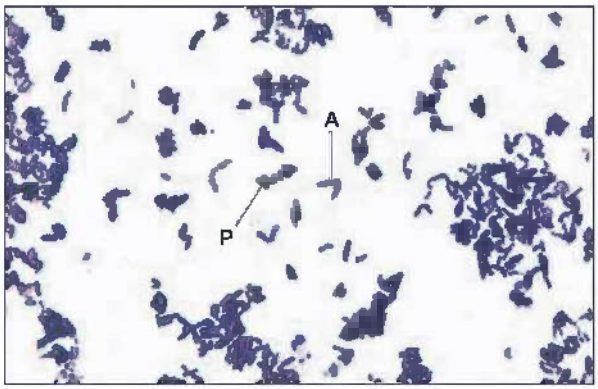
3.78 Streptococcus Arrangement (Gram Stain) ♦ As its name suggests, *Streptococcus salivarius* forms chains of spherical cells and it inhabits the oral cavity, but primarily the saliva and tongue. This specimen is from a broth culture (which enables the cells to form long chains) and was stained with crystal violet. Individual cells are approximately 1 µm in diameter.



3.79 Staphylococcus Arrangement (Gram Stain) ♦ *Staphylococcus aureus* cells grown in broth culture are shown. Note that the staphylococcal groups are rarely bigger than a dozen cells or so. Larger clusters are probably two or more groups that happen to be next to each other on the slide. This is especially problematic when making a preparation of cells grown on a solid medium. Be sure to emulsify them thoroughly to spread clusters out as much as possible. *S. aureus* is a common opportunistic pathogen of humans. Cells are approximately 1 µm in diameter.



3.80 Streptobacillus Arrangement (Crystal Violet Stain) ♦ *Bacillus megaterium* is a streptobacillus. These cells were obtained from culture and are 2 µm–5 µm long by 1.2 µm–1.5 µm wide.



3.81 Palisade and Angular Arrangements of *Corynebacterium* (Gram Stain) ♦ Stacking of rod-shaped cells side-by-side is the palisade arrangement (P). An angular arrangement (A) is when a pair of rods is bent where the cells join. Both arrangements are due to "snapping division" in which the cells divide lengthwise and remain more or less attached. *Anthrobacter* also shows these arrangements.

EXERCISE
3-5

Simple Stains

Theory

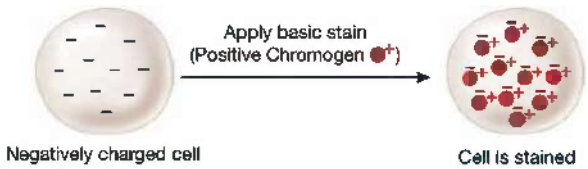
Stains are solutions consisting of a solvent (usually water or ethanol) and a colored molecule (often a benzene derivative), the **chromogen**. The portion of the chromogen that gives it its color is the **chromophore**. A chromogen may have multiple chromophores, with each adding intensity to the color. The **auxochrome** is the charged portion of a chromogen and allows it to act as a dye through ionic or covalent bonds between the chromogen and the cell. **Basic stains**[1] (where the auxochrome becomes positively charged as a result of picking up a hydrogen ion or losing a hydroxide ion) are attracted to the negative charges on the surface of most bacterial cells. Thus, the cell becomes colored (Fig. 3.82). Common basic stains include methylene blue, crystal violet, and safranin. Examples of basic stains may be seen in Figures 3.69, 3.80, and 3.83.

Basic stains are applied to bacterial smears that have been **heat-fixed**. Heat-fixing kills the bacteria, makes them adhere to the slide, and coagulates cytoplasmic proteins to make them more visible. It also distorts the cells to some extent.

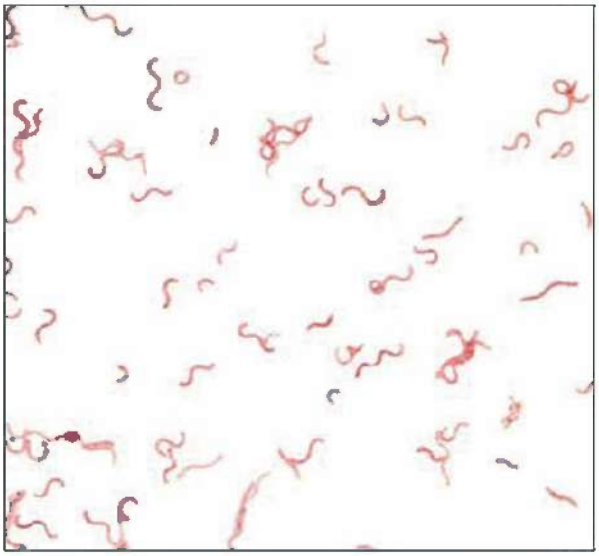
Application

Because cytoplasm is transparent, cells usually are stained with a colored dye to make them more visible under the microscope. Then cell morphology, size, and arrangement can be determined. In a medical laboratory, these are usually determined with a Gram stain (Exercise 3-7), but you will be using simple stains as an introduction to these.

[1] Notice that the term *basic* means "alkaline," not "elementary"; however, coincidentally, *basic* stains can be used for *simple* staining procedures.



3.82 Chemistry of Basic Stains ♦ Basic stains have a positively charged chromogen (●⁺), which forms an ionic bond with the negatively charged bacterial cell, thus colorizing the cell.



3.83 Safranin Dye in a Simple Stain ♦ This is a simple stain using safranin, a basic stain. Notice that the stain is associated with the cells and not the background. The organism is *Rhodospirillum rubrum* grown in broth culture.

In This Exercise

Today you will learn how to prepare a bacterial emulsion (smear) and perform simple stains. Several different organisms will be supplied so you can begin to see the variety of cell morphologies and arrangements in the bacterial world. We suggest that you perform all the stains on one or two organisms (to get practice) and look at your lab partners' stains to see the variety of cell types. Be sure that you view all the available organisms.

✓ Materials

Per Student Group

- ☐ Clean glass microscope slides
- ☐ Methylene blue stain
- ☐ Safranin stain
- ☐ Crystal violet stain
- ☐ Disposable gloves
- ☐ Chemical eye protection
- ☐ Squirt bottle with water
- ☐ Staining tray

- ☐ Staining screen
- ☐ Bibulous paper (or paper towels)
- ☐ Slide holder
- ☐ Compound microscope with oil-immersion lens and ocular micrometer
- ☐ Immersion oil
- ☐ Lens paper
- ☐ Recommended organisms:
  - *Bacillus cereus*
  - *Micrococcus luteus*
  - *Neisseria sicca*
  - *Rhodospirillum rubrum*
  - *Staphylococcus epidermidis*
  - *Vibrio* spp.

Procedure

1. A bacterial emulsion (smear) is made prior to most staining procedures. Follow the procedural diagram in Figure 3.84 to prepare smears of two different organisms on a slide. Be sure not to mix them. Make a second and third slide with the remaining four organisms at the same time so they can be air drying simultaneously. Emulsions should be about the size of a dime.
2. Heat-fix each slide as described in Figure 3.84. If you are using a bacterial incinerator, hold the slide (with a slide holder) near the opening for a few seconds (Fig. 3.85).
3. Following the basic staining procedure illustrated in the procedural diagram in Figure 3.86, stain one of your slides with each stain using the following times:

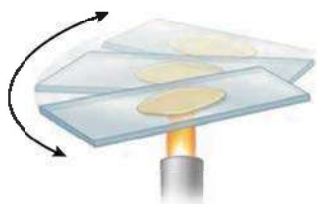
crystal violet:	stain for 30 to 60 seconds
safranin:	stain for up to 1 minute
methylene blue:	stain for 30 to 60 seconds

   Be sure to wear gloves when staining. Record your actual staining times in the table provided in the data sheet, page 189, so you can adjust for over- or under-staining.
4. Using the oil-immersion lens, observe each slide. Record your observations of cell morphology, arrangement, and size in the chart provided on the data sheet.
5. Dispose of the slides and used stain according to your laboratory's policy.

1. Place a small drop of water (not too much) on a clean slide using an inoculating loop. If you are staining from a broth culture, begin with Step 2.

2. Aseptically add bacteria to the water. ***Note:*** If you find that you are transferring too much organism with a loop, use an inoculating needle. If you are transferring a BSL-2 organism, use a sterile wooden stick or a disposable loop and dispose of it properly when finished. Up to three emulsions can comfortably be made on a single slide. Mix in the bacteria and spread the drop out. Avoid spattering the emulsion as you mix. Flame your loop when done.

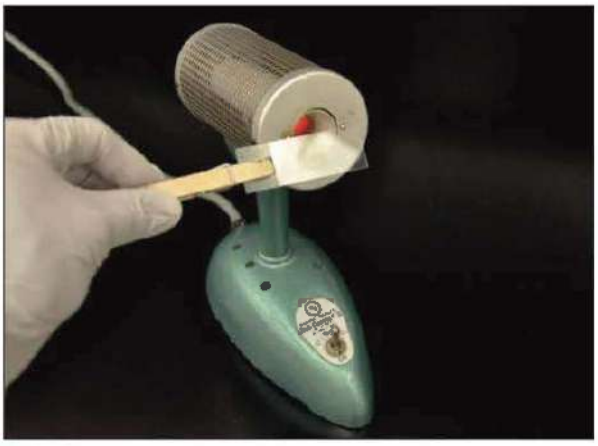
3. Allow the smear to air dry. If prepared correctly, the smear should be slightly cloudy.



4. Using a slide holder, pass the smear through the upper part of a flame two or three times. This heat-fixes the preparation. Avoid overheating the slide because aerosols may be produced.

5. Allow the slide to cool, then continue with the staining protocol.

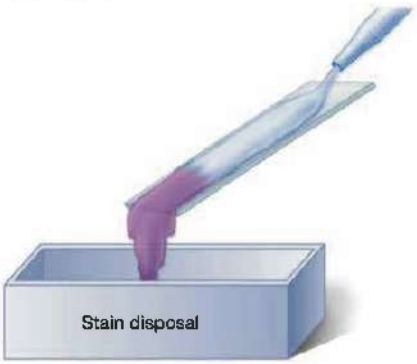
3.84 Procedural Diagram: Making a Bacterial Smear (Emulsion) ♦ You will apply this technique as the first step in performing most stain procedures (the negative and capsule stains are the exceptions). It is an important skill to learn, because preparation of uniform bacterial smears will make it easier to obtain consistent staining results. ***Caution:*** Avoid producing aerosols. Do not spatter the smear as you mix it, do not blow on or wave the slide to speed up air-drying, and do not overheat when heat-fixing. If working with a BSL-2 organism, use a wooden stick or disposable loop to transfer the organism to the slide and heat-fix by holding the slide near the opening of a bacterial incinerator (Fig. 1.14).



3.85 Heat-fixing a BSL-2 Organism ♦ In order to reduce aerosol production, heat-fixing a slide can be done by holding it near the opening of a microincinerator or over the grill for 20–30 seconds. This is an absolute requirement if staining a BSL-2 organism.

1. Begin with a heat-fixed emulsion (see Figure 3-84). More than one organism can be put on a slide. In this exercise, we recommend putting three organisms on each slide.

2. Wearing gloves, place the slide on a rack over a staining tray. Cover the smear(s) with the stain. Make sure any excess stain falls into the staining tray.



3. Grasp the slide with a slide holder and hold it on an angle. Gently rinse the slide with distilled water into the staining tray. Dispose of stain in the tray at the end of lab according to your lab's practices.

4. Gently blot dry in a tablet of bibulous paper or paper towels. (Alternatively, a page from the tablet can be removed and used for blotting.) Do not rub. When dry, observe under oil immersion.

3.86 Procedural Diagram: Simple Stain ♦ Staining times differ for each stain, but cell density of your smear also affects staining time. Strive for consistency in making your smears. You also only need to cover the emulsion(s) on the slide, not the slide's whole surface. ***Caution***: Be sure to flame your loop after cell transfer and properly dispose of the slide when you are finished observing it.

References

Chapin, Kimberle. Chap. 4 in *Manual of Clinical Microbiology*, 6th ed. Patrick R. Murray, Ellen Jo Baron, Michael A. Pfaller, Fred C. Tenover, and Robert H. Yolken, eds. Washington, DC: American Society for Microbiology, 1995.

Chapin, Kimberle C. and Patrick R. Murray. Pages 257–259 in *Manual of Clinical Microbiology*, 8th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Michael A. Pfaller, and Robert H. Yolken, eds. Washington, DC: American Society for Microbiology, 2003.

Murray, R. G. E., Raymond N. Doetsch, and C. F. Robinow. Page 27 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Norris, J. R. and Helen Swain. Chap. II in *Methods in Microbiology*, Vol. 5A. J. R. Norris and D. W. Ribbons, eds. London, UK: Academic Press, Ltd., 1971.

Power, David A. and Peggy J. McCuen. Page 4 in *Manual of BBL™ Products and Laboratory Procedures*, 6th ed. Cockeysville, MD: Becton Dickinson Microbiology Systems, 1988.

Tille, Patricia M. Pages 70–71 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-5

Simple Stains

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations in the table below.

Organism	Stain and Duration	Cellular Morphology and Arrangement (include a sketch)	Cell Dimensions

QUESTIONS

1 *What are some consequences of leaving a stain on a bacterial smear too long (over-staining)?*

2 *What are some consequences of not leaving a stain on a smear long enough (under-staining)?*

3 *Choose a coccus and a bacillus from the organisms you observed and calculate their surface-to-volume ratios. Consider the coccus to be a perfect sphere and the bacillus to be a cylinder. Use the equations supplied.*

Cell Morphology	Surface Area	Volume
Coccus	$SA = 4\pi r^2$	$SA = \frac{4}{3}\pi r^2$
Bacillus	$SA = 2\pi rh + 2\pi r^2$	$V = \pi r^2 h$

r = radius, h = height, $\pi = 3.14$

Surface-to-Volume Ratio of Sample Cells

Organism	Cell Morphology	Surface Area (μm^2)	Volume (μm^3)	Surface-to-Volume Ratio

4 *Consider a coccus and a rod of equal volume.*

a. *Which is more likely to survive in a dry environment? Explain your answer.*

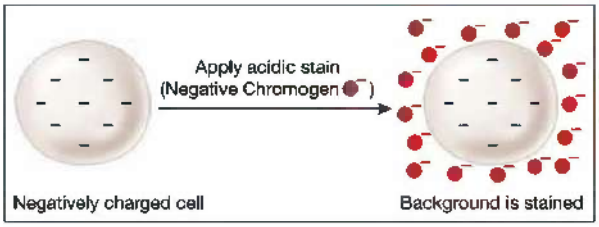
b. *Which would be better adapted to a moist environment? Explain your answer.*

EXERCISE
3-6

Negative Stains

Theory

The negative staining technique uses a dye solution in which the chromogen is acidic and carries a negative charge. (An acidic chromogen gives up a hydrogen ion, which leaves it with a negative charge.) The negative charge on the bacterial surface repels the negatively charged chromogen, so the cell remains unstained against a colored background (Fig. 3.87). A specimen stained with the acidic stain nigrosin is shown in Figure 3.88.



3.87 Chemistry of Acidic Stains ◆ Acidic stains have a negatively charged chromogen ($\bullet^-$) that is repelled by negatively charged cells. Thus, the background is colored and the cell remains transparent.

Application

The negative staining technique is used to determine morphology and cellular arrangement in bacteria that are too delicate to withstand heat-fixing. A primary example is the spirochete *Treponema*, which is distorted by the heat-fixing of other staining techniques. Also, where determining the accurate size is crucial, a negative stain can be used because it produces minimal cell shrinkage.

In This Exercise

Today you will perform negative stains on three different organisms. You also will have the opportunity to compare the sizes of *Bacillus cereus*, *Micrococcus luteus*, and *Rhodospirillum rubrum* measured with the negative stain to their sizes as determined using a simple stain.

3.88 Nigrosin Negative Stain ◆ Notice that the *Bacillus megaterium* cells are unstained against a dark background. The circular objects are bubbles.

✓ Materials

Per Student Group

- ☐ Nigrosin stain or eosin stain
- ☐ Clean glass microscope slides
- ☐ Disposable gloves
- ☐ Chemical eye protection
- ☐ Compound microscope with oil-immersion lens and ocular micrometer
- ☐ Immersion oil
- ☐ Lens paper
- ☐ Bibulous paper or paper towel
- ☐ Recommended organisms:
  - *Bacillus cereus*
  - *Micrococcus luteus*
  - *Rhodospirillum rubrum*

Procedure

1. Follow the procedural diagram in Figure 3.89 to prepare a negative stain of each organism. Do NOT heat-fix your slides after air-drying them.
2. Dispose of the spreader slide in a disinfectant jar or sharps container immediately after use.
3. Observe using the oil-immersion lens. Record your observations in the chart on the data sheet, page 193.
4. Dispose of the specimen slide in a disinfectant jar or sharps container after use.

1. Begin with a drop of acidic stain at one end of a clean slide. Be sure to wear gloves.

2. Working on the table top covered with a paper towel, aseptically add organisms and emulsify with the loop. Don't over-inoculate and avoid spattering the mixture. Sterilize the loop after emulsifying. ***Note:*** If you find you are transferring too much organism with a loop, use an inoculating needle. If you are transferring a BSL-2 organism, use a sterile wooden stick or disposable loop/needle and dispose of it properly when finished.

3. Take a second clean slide, place it on the surface of the first slide, and draw it back into the drop.

4. When the drop flows across the width of the spreader slide...

5. ...push the spreader slide to the other end. Dispose of the spreader slide in a jar of disinfectant or sharps container.

6. Air-dry and observe under the microscope. Do NOT heat-fix.

3.89 Procedural Diagram: Negative Stain ◆ Be sure to sterilize your loop after transfer and to appropriately dispose of the spreader slide immediately after use. Do not set it on the table. Once air-dried, the slide is ready for viewing. No heat-fixing is required in this procedure.

References

Claus, G. William. Chap. 5 in *Understanding Microbes—A Laboratory Textbook for Microbiology*. New York: W. H. Freeman and Co., 1989.

Murray, R. G. E., Raymond N. Doetsch, and C. F. Robinow. Page 27 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-6

Negative Stains

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations in the table below.

Organism	Stain	Cellular Morphology and Arrangement (include a detailed sketch of a few representative cells)	Cell Dimensions from Negative Stain	Cell Dimensions from Simple Stain

QUESTIONS

1 *Why doesn't a negative stain colorize the cells in the smear?*

2 *Eosin is a red stain and methylene blue is blue. What should be the result of staining a bacterial smear with a mixture of eosin and methylene blue?*

3

3 *Compare the sizes of* B. cereus, M. luteus, *and* R. rubrum *cells as measured using a basic stain (Exercise 3-5) and an acidic stain. What might account for any difference?*

Differential and Structural Stains

Differential stains allow a microbiologist to detect differences between organisms or differences between parts of the same organism. In practice, these are used much more frequently than simple and negative stains because they not only allow determination of cell size, morphology, and arrangement (as with simple or negative stains) but provide information about other features as well.

The Gram stain is the most commonly used differential stain in bacteriology. Other differential stains are used for organisms not distinguishable by the Gram stain and for those that have other important cellular attributes, such as acid-fastness, a capsule, spores, or flagella. With the exception of the acid-fast stain, these other stains sometimes are referred to as **structural stains.** ◆

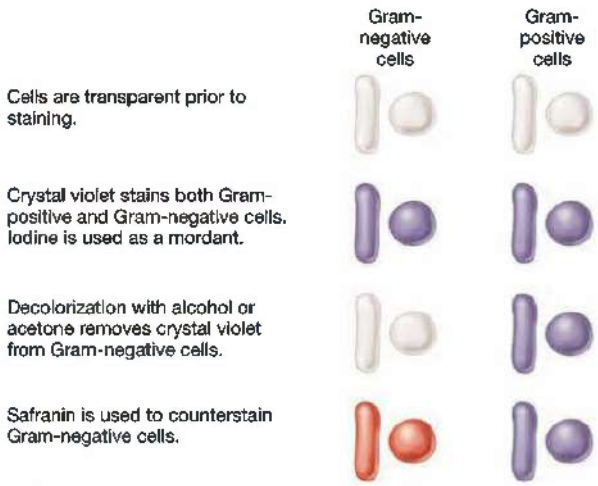
EXERCISE 3-7

Gram Stain

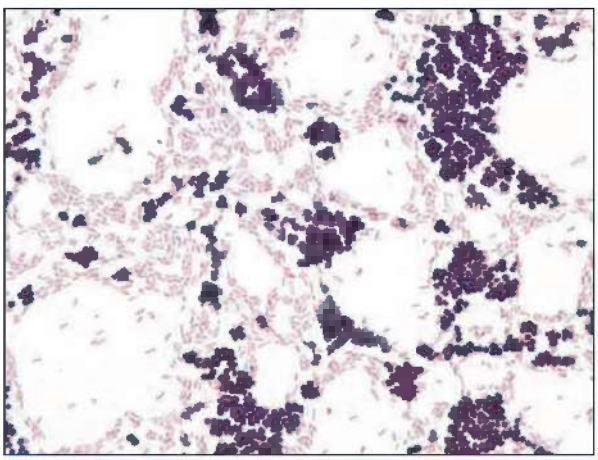
Theory

The Gram stain is a differential stain in which a **decolorization** step occurs between the applications of two basic stains. The Gram stain has many variations, but they all work in basically the same way (Fig. 3.90). The **primary stain** is crystal violet. Iodine is added as a **mordant** to enhance crystal violet staining by forming a **crystal violet–iodine complex.** Decolorization follows and is the most critical step in the procedure. Gram-negative cells are decolorized by the solution (generally an alcohol/acetone mixture of varying proportions) whereas Gram-positive cells are not. Gram-negative cells can thus be colorized by the red **counterstain** safranin, but Gram-positive cells are already violet and cannot. Upon successful completion of a Gram stain, Gram-positive cells appear purple and Gram-negative cells appear reddish-pink (Fig. 3.91).

Electron microscopy and other evidence indicate that the ability to resist decolorization or not is based on the different wall constructions of Gram-positive and


Gram-negative cells
Gram-positive cells
Cells are transparent prior to staining.
Crystal violet stains both Gram-positive and Gram-negative cells. Iodine is used as a mordant.
Decolorization with alcohol or acetone removes crystal violet from Gram-negative cells.
Safranin is used to counterstain Gram-negative cells.

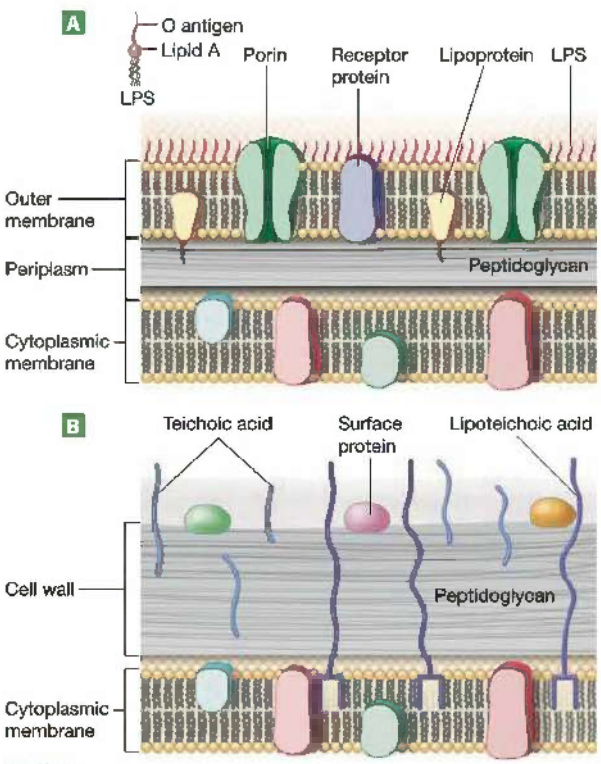
3.90 Gram Stain Overview ◆ After application of the primary stain (crystal violet), decolorization, and counterstaining with safranin, Gram-positive cells stain violet and Gram-negative cells stain pink/red. Notice that crystal violet and safranin are both basic stains, and that the decolorization step is what makes the Gram-stain differential.



3.91 Micrograph of Two Species Illustrating Gram Stain Results ◆ The violet staphylococcal cells are Gram positive; the pink rods are Gram negative. Depending on your Gram-stain kit, the safranin may be anywhere from a light pink (as in this micrograph) to a more intense reddish color. In either case, it should be easily distinguishable from the crystal violet color.

Gram-negative cells. Gram-negative cell walls have a higher lipid content (because of the outer membrane) and a thinner peptidoglycan layer than Gram-positive cell walls (Fig. 3.92). The alcohol/acetone in the decolorizer extracts the lipid, making the Gram-negative wall more porous and incapable of retaining the crystal violet–iodine complex, thereby decolorizing it. The thicker peptidoglycan and greater degree of cross-linking (because of teichoic acids) trap the crystal violet–iodine complex more effectively, making the Gram-positive wall less susceptible to decolorization.

Although some organisms give Gram-variable results, most variable results are a consequence of poor technique. The decolorization step is the most crucial and most likely source of Gram-stain inconsistency. It is possible to **over-decolorize** by leaving the decolorizer on too long and get reddish Gram-*positive* cells. It also is possible to **under-decolorize** and produce purple Gram-*negative* cells.



3.92 Bacterial Cell Walls ♦ The Gram-negative wall (**A**) is composed of less peptidoglycan (as little as a single layer) and more lipid (due to the outer membrane) than the Gram-positive wall (**B**). Though it is shown as a solid layer, the peptidoglycan is quite porous. The thicker Gram-positive peptidoglycan is like a three-dimensional chain-link fence, and when the crystal violet–iodine complexes form within it, they are difficult to remove by decolorization. The greater amount of lipid and thinner peptidoglycan of the Gram-negative wall makes extraction of the crystal violet–iodine complex by the decolorizer much easier.

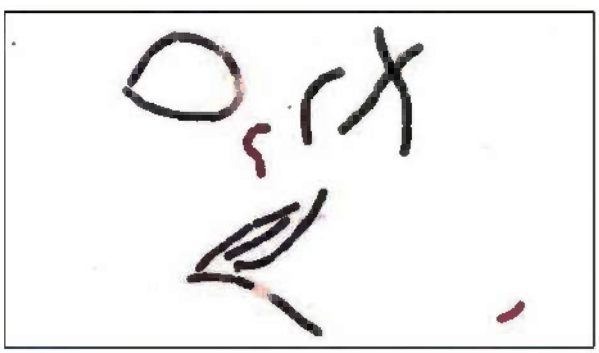
Neither of these situations changes the actual Gram reaction for the organism being stained. Rather, these are false results because of poor technique.

A second source of poor Gram stains is inconsistency in preparation of the emulsion. Remember, a good emulsion is about dime size and dries to a faint haze on the slide.

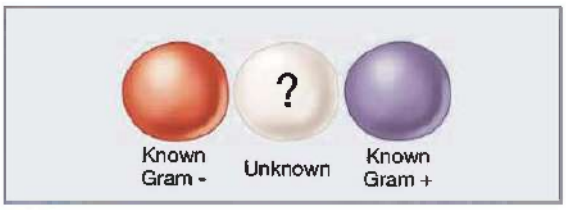
A third source of inconsistent Gram stains may be the organisms themselves. Some Gram positives, especially *Bacillus* and *Staphylococcus* species, lose their ability to retain the crystal violet–iodine complex in as little as 24 hours of incubation. Figure 3.93 illustrates a *Bacillus* culture stained at 24 hours of growth. Always plan on doing your Gram stains on cultures no older than 24 hours for best results.

Until correct results are obtained consistently, it is recommended that control smears of Gram-positive and Gram-negative organisms be stained along with the organism in question (Fig. 3.94). As an alternative control, a direct smear made from the gumline may be Gram stained (Fig. 3.95) with the expectation that both Gram-positive and Gram-negative organisms will be seen. Over-decolorized and under-decolorized gumline direct smears are shown for comparison (Figs. 3.96 and 3.97). Positive controls also should be run when using new reagent batches.

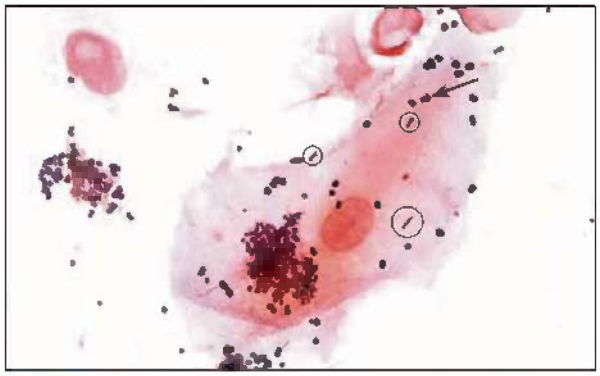
Interpretation of Gram stains can be complicated by nonbacterial elements. For instance, stain crystals from an old or improperly made stain solution can disrupt the field (Fig. 3.98) or stain precipitate may be mistakenly identified as bacteria (Fig. 3.99).



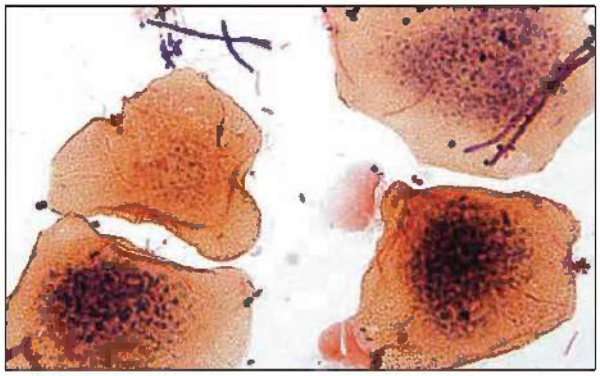
3.93 24-Hour *Bacillus* Illustrating Loss of Gram-Positive Reaction with Age ♦ This *Bacillus* was isolated from aerobic estuarine mud and stained within 24 hours of making the pure culture. Notice some cells are pink and have lost their ability to resist decolorization. This result does not change the fact that *Bacillus* has a Gram-positive wall, though.



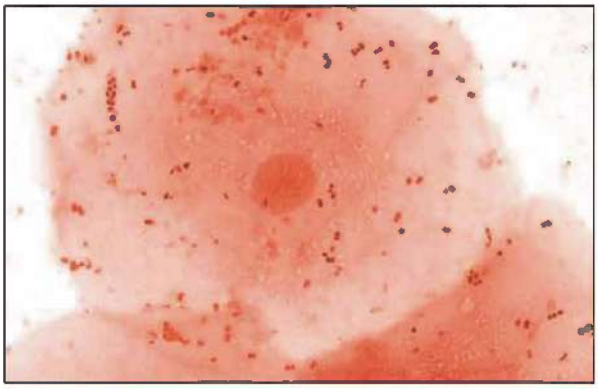
3.94 Positive Controls to Check Your Technique ◆ Staining known Gram-positive and Gram-negative organisms on either side of your unknown organism act as positive controls for your technique. Try to make the emulsions as close to one another as possible. Spreading them out across the slide makes it difficult to stain and decolorize them equally. It is also beneficial to put the Gram-negative control at the end where you will be applying the decolorizer. This will make it easier to see when it is running clear. Putting it at the other end means it will have runoff from the Gram-positive control and the unknown obscuring it.



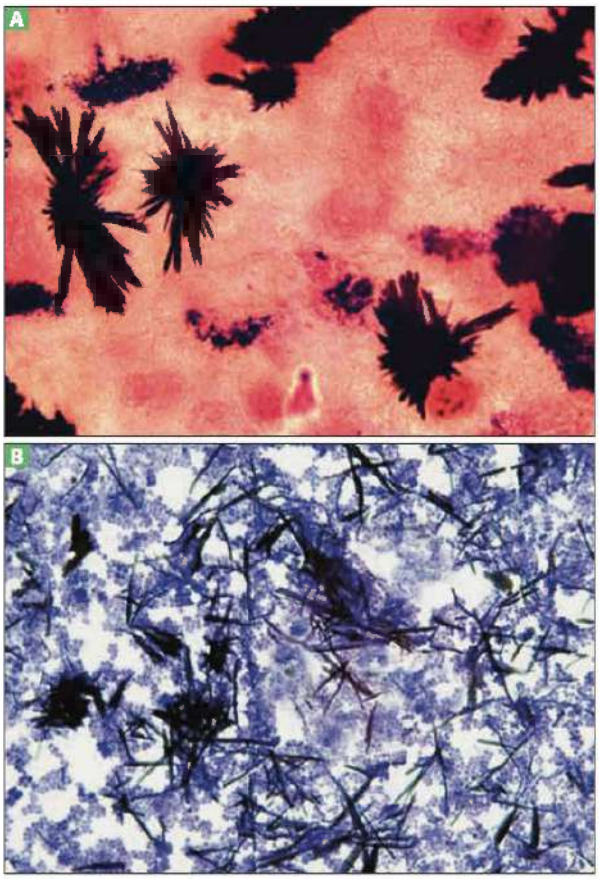
3.95 Direct Smear Positive Control (Gram Stain) ◆ A direct smear made from the gumline may also be used as a Gram-stain control. Expect numerous Gram-positive bacteria (especially cocci) and some Gram-negative cells, including your own epithelial cells. In this slide, Gram-positive cocci predominate, but a few Gram-negative cells are visible, including Gram-negative rods (circled) and a Gram-negative diplococcus (arrow). Notice that most bacterial cells are on the epithelial cell's surface, which is where they reside when in the mouth.



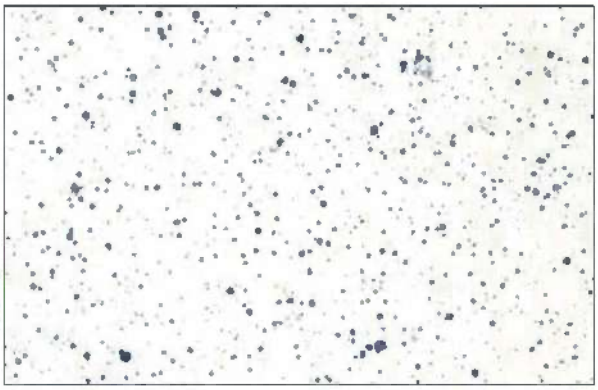
3.96 Under-Decolorized Gram Stain ◆ This is a direct smear from the gumline. Notice the purple patches of stain on the epithelial cells. Also notice the variable quality of this stain—the epithelial cell to the left of center is stained better than the others.



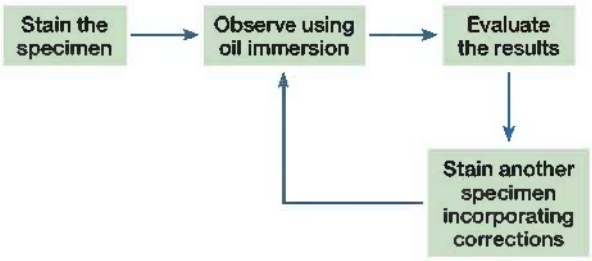
3.97 Over-Decolorized Gram Stain ◆ This also is a direct smear from the gumline. Notice the virtual absence of any purple cells, a certain indication of over-decolorization.



3.98 Crystal-Violet Crystals (Gram Stain) ◆ If the staining solution is not adequately filtered or is old, crystal-violet crystals may appear. Although they are pleasing to the eye, they obstruct your view of the specimen. Crystals from two different Gram stains are shown here. (A) This is a gumline direct smear. The bacterial cells are the pink material in the background and the crystals are the large, purple "snowflakes." (B) In this Gram stain of *Micrococcus roseus* grown in culture, the cells are the lighter purple in the background (can you detect any tetrads?) and the crystals are the darker spikes.



3.99 Stain Precipitate (Gram Stain) ◆ If the slide is not rinsed thoroughly or the stain is allowed to dry on the slide, spots of stain precipitate may form and may be confused with bacterial cells. Their variability in size is a clue that they are not bacteria.



3.100 Evaluating Staining Technique ◆ It is best when learning a staining technique to prepare several slides and stain them one at a time. Observe the first slide, evaluate its quality (good or poor) and if poor, determine what steps to take to improve the quality. Then, stain another slide incorporating the changes suggested by your previous attempt. This technique is applicable to any stain, not just the Gram stain.

Application

The Gram stain, used to distinguish between Gram-positive and Gram-negative cells, is the most important and widely used microbiological differential stain. In addition to Gram reaction, this stain allows determination of cell morphology, size, and arrangement. It typically is the first differential test run on a specimen brought into the laboratory for identification. In some cases, a rapid, presumptive identification of the organism or elimination of a particular organism is possible.

In This Exercise

The Gram stain is the single most important differential stain in bacteriology. Therefore, you will have to practice it and practice it some more to become proficient in its execution. The organisms should be used in the combinations given so you will have one Gram-positive and one Gram-negative on each slide. We recommend that you stain one slide, observe it, evaluate it, and then stain another slide incorporating corrections indicated by the first slide's results (Fig. 3.100).

✓ Materials

Per Student Group

- ☐ Compound microscope with oil-immersion lens and ocular micrometer
- ☐ Clean glass microscope slides
- ☐ Sterile toothpick
- ☐ Gram-stain solutions (commercial kits are available):
  - Gram crystal violet
  - Gram iodine
  - 95% ethanol (or ethanol/acetone solution)
  - Gram safranin
- ☐ Squirt bottle with water
- ☐ Bibulous paper or paper towels
- ☐ Disposable gloves
- ☐ Chemical eye protection
- ☐ Staining tray
- ☐ Staining screen
- ☐ Slide holder
- ☐ Recommended organisms (overnight cultures grown on agar slants or turbid broth cultures):
  - *Staphylococcus epidermidis*
  - *Escherichia coli*
  - *Neisseria sicca*
  - *Corynebacterium xerosis*

Procedure

1. Follow the procedure illustrated in Figure 3.84 to prepare and heat-fix smears of *Staphylococcus epidermidis* and *Escherichia coli* immediately next to one another on the same clean glass slide. (If you make the emulsions at opposite ends of the slide, you may find it difficult to stain and decolorize each equally.) Strive to prepare smears of uniform thickness, because thick smears risk being under-decolorized.
2. Repeat step 1 for *Neisseria sicca* and *Corynebacterium xerosis* on a second slide.
3. Because Gram stains require much practice, we recommend that you prepare several slides of each combination and let them air-dry simultaneously. Then they will be ready when you need them.

4. Wash your hands, and then use the sterile toothpick to obtain a sample from your teeth at the gumline. (Do not draw blood! What you want is easily removed from your gingival pockets.) Transfer the sample to a drop of water on a clean glass slide, air-dry, and heat-fix.
5. Follow the basic staining procedure illustrated in Figure 3.101. We recommend staining the pure cultures first. After your technique is consistent, stain the oral sample. Be sure to wear gloves. Also, pay special attention to the decolorization "tips" in step 6 of Figure 3.101 and in its caption.
6. Observe using the oil-immersion lens. Record your observations of cell morphology and arrangement, dimensions, and Gram reactions in the chart provided on the data sheet, page 201.
7. Dispose of the specimen slides in a jar of disinfectant or a sharps container after use.

References

Atlas, Ronald M. and James W. Snyder. Pages 278–279 in *Manual of Clinical Microbiology*, 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Chapin, Kimberle C. and Patrick R. Murray. Pages 258–260 in *Manual of Clinical Microbiology*, 8th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Michael A. Pfaller, and Robert H. Yolken, eds. Washington, DC: American Society for Microbiology, 2003.

Koneman, Elmer W., Stephen D. Allen, William M. Janda, Paul C. Schreckenberger, and Washington C. Winn, Jr. Chap. 14 in *Color Atlas and Textbook of Diagnostic Microbiology*, 5th ed. Philadelphia: J. B. Lippincott Co., 1997.

Murray, R. G. E., Raymond N. Doetsch, and C. F. Robinow. Pages 31 and 32 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Norris, J. R., and Helen Swain. Chap. II in *Methods in Microbiology*, Vol. 5A. J. R. Norris and D. W. Ribbons, eds. London, UK: Academic Press, Ltd., 1971.

Power, David A. and Peggy J. McCuen. Page 261 in *Manual of BBL™ Products and Laboratory Procedures*, 6th ed. Cockeysville, MD: Becton Dickinson Microbiology Systems, 1988.

Tille, Patricia M. Pages 70–73 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, Inc., an affiliate of Elsevier, Inc. 2014.

1. Begin with up to three heat-fixed emulsions on one slide. (See Figure 3.84.) Wearing gloves, place the slide on a rack over a staining tray.

2. Cover the smear(s) (not the whole slide) with crystal violet stain for one minute. Be sure the staining tray catches all excess stain.

3. Grasp the slide with a slide holder and hold it on an angle. Gently rinse the slide with distilled water into the staining tray. Alternatively, see steps 4 and 5.

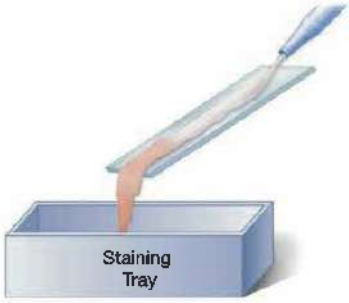
4. Cover the smear(s) with Gram iodine stain for one minute. Be sure the staining tray catches all excess stain.

5. Grasp the slide with a slide holder and hold it on an angle. Gently rinse the slide with distilled water into the staining tray.

6. Holding the slide on an angle, decolorize with Gram decolorizer by allowing it to trickle down the slide until the runoff is clear. Catch the runoff in the staining tray. ***Note:*** Decolorizers differ depending on the kit's manufacturer. The more acetone in the decolorizer, the faster it will decolorize.

7. As soon as the runoff is clear, IMMEDIATELY rinse the slide with diluted water into the staining tray (***Note:*** It will still be decolorizing while you're grabbing the water bottle! Plan ahead for that transition.).

8. Counterstain the smear(s) with safranin for one minute. Be sure the staining tray catches all excess stain.



9. Grasp the slide with a slide holder and hold it on an angle. Gently rinse the slide with distilled water into the staining tray.

10. Gently blot dry in a tablet of bibulous paper or paper towels. (Alternatively, a page from the tablet can be removed and used for blotting.) Do not rub. When dry, observe under oil immersion.

3.101 Procedural Diagram: Gram Stain ◆ Pay careful attention to the staining times. If your preparations do not give "correct" results, the most likely source of error is in the decolorization step. When the runoff is clear (generally within 10 seconds, shorter if the acetone concentration is high), IMMEDIATELY rinse with dH_2O. *The decolorizer continues decolorizing as you put its bottle down and pick up the water bottle, so anticipate the end point of decolorization to compensate for this.*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-7

Gram Stain

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations in the table below. Use separate lines for different organisms found in the Gram stain of your gumline. Include a drawing of your own epithelial cells.

Organism of Source	Cellular Morphology and Arrangement (include a detailed sketch of a few representative cells)	Cell Dimensions	Color	Gram Reaction (+/−)

QUESTIONS

1 *Predict the effect on Gram-positive and Gram-negative cells of the following "mistakes" made when performing a Gram stain. Consider each mistake independently.*

a. *Failure to add the iodine.*

b. *Failure to apply the decolorizer.*

c. *Failure to apply the safranin.*

d. *Reversal of crystal violet and safranin stains.*

2 *Both crystal violet and safranin are basic stains and may be used to do simple stains on Gram-positive and Gram-negative cells. This being the case, explain how they end up staining Gram-positive and Gram-negative cells differently in the Gram stain.*

3 *If you saw large, eukaryotic cells in the preparation made from your gumline, they were most likely your own epithelial cells. Are you Gram-positive or Gram-negative? (You can make a good guess about this even if you didn't see your cells.)*

4 *One of your lab partners has followed the recommended procedure of running Gram-positive and Gram-negative control organisms on her Gram stain of an unknown species. Her choices of controls were* Escherichia coli *and* Bacillus subtilis. *She tries several times and each time concludes she is decolorizing too long because both controls have pink cells (one more than the other). What might you suggest she try and why?*

EXERCISE
3-8

Acid-Fast Stains

Theory

The presence of mycolic acids in the cell walls of acid-fast organisms is the cytological basis for the acid-fast differential stain. Mycolic acid is a waxy substance that gives acid-fast cells a higher affinity for the primary stain and resistance to decolorization by an acid alcohol solution (3% HCl in 95% ethanol).

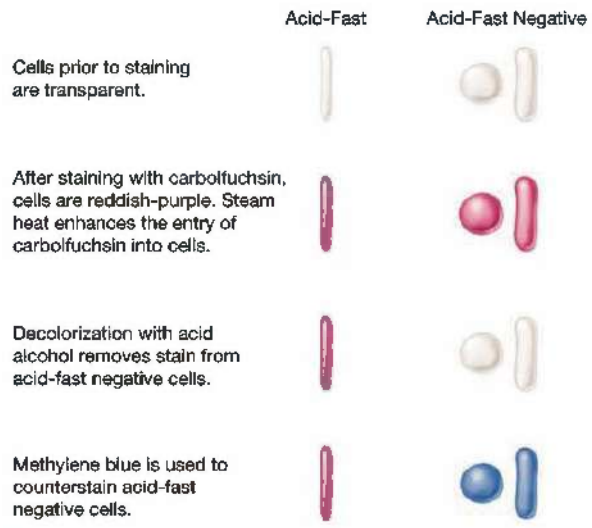
A variety of acid-fast staining procedures are employed, two of which are the Ziehl-Neelsen (ZN) method and the Kinyoun (K) method. These differ primarily in that the ZN method uses heat as part of the staining process (this is in addition to heat-fixing the preparation), whereas the K method is a "cold" stain (which is still heat-fixed). In both protocols the bacterial smear may be prepared in a drop of serum to help the "slippery" acid-fast cells (they are waxy, after all) adhere to the slide. The two methods provide comparable results and the choice of method comes down to lab conventions and personal preference.

The waxy wall of acid-fast cells repels typical aqueous stains. (As a result, most acid-fast positive organisms are only weakly Gram positive.) In the ZN method (Fig. 3.102), the phenolic compound carbolfuchsin is used as the primary stain because it is lipid soluble and penetrates the waxy cell wall. Staining by carbolfuchsin is further enhanced by steam heating the preparation to melt the wax and allow the stain to move into the wall. Acid alcohol is used to decolorize nonacid-fast cells; acid-fast cells resist this decolorization. A counterstain, such as methylene blue, then is applied. Acid-fast cells are reddish-purple; nonacid-fast cells are blue (Fig. 3.103).

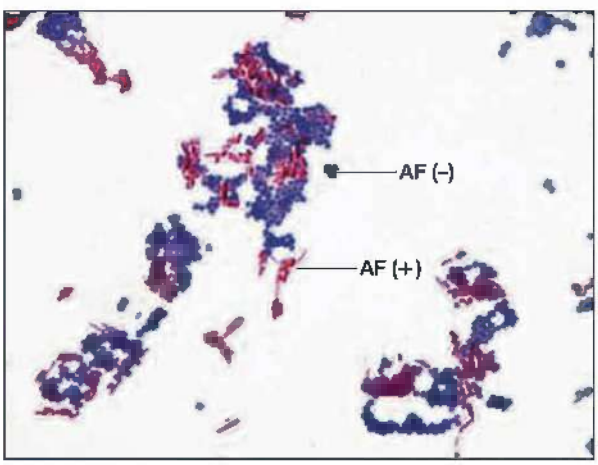
The Kinyoun method (Fig. 3.104) uses a slightly more lipid-soluble and concentrated carbolfuchsin as the primary stain. These properties allow the stain to penetrate the acid-fast walls without the use of heat but make this method slightly less sensitive than the ZN method. Decolorization with acid alcohol is followed by a contrasting counterstain, such as brilliant green (Fig. 3.105) or methylene blue.

Application

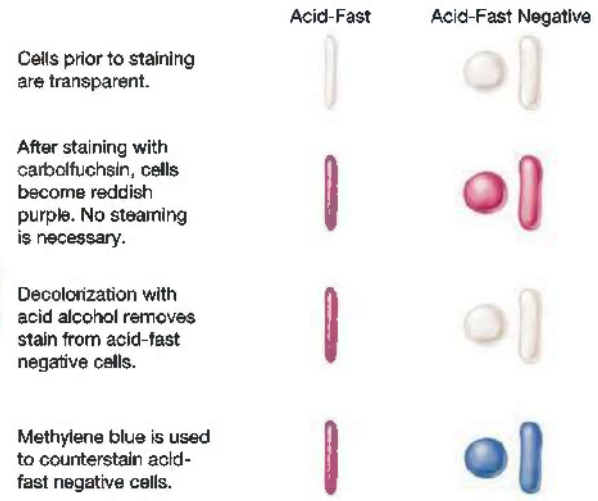
The acid-fast stain is a differential stain used to detect cells capable of retaining a primary stain when treated with an acid alcohol. It is an important differential stain used to identify bacteria in the genus *Mycobacterium*, some of which are pathogens (e.g., *M. leprae* and *M. tuberculosis*, causative agents of leprosy and tuberculosis, respectively).


Acid-Fast
Acid-Fast Negative
Cells prior to staining are transparent.
After staining with carbolfuchsin, cells are reddish-purple. Steam heat enhances the entry of carbolfuchsin into cells.
Decolorization with acid alcohol removes stain from acid-fast negative cells.
Methylene blue is used to counterstain acid-fast negative cells.

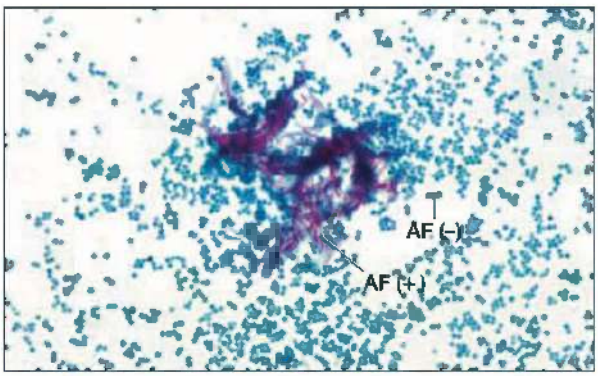
3.102 Ziehl-Neelsen Acid-Fast Stain Overview ◆ Acid-fast cells stain reddish purple; acid-fast negative cells stain blue or the color of the counterstain if a different one is used.



3.103 Acid-Fast Stain Using the ZN Method ◆ Notice how most of the *Mycobacterium phlei* (AF +) cells are in clumps, an unusual state for most rods. They do this because their waxy cell walls make them sticky. Try carefully and gently mixing them a little longer when preparing the slide than you would for other stains. A few individual cells are visible, however, and they clearly are rods that measure 0.5 µm by 2–3 µm. The *Staphylococcus epidermidis* cells (AF –) are also in clumps, but that is because they grow as grapelike clusters. Each cell's diameter is approximately 1µm. Compare this micrograph with Figure 3.105.


Acid-Fast
Acid-Fast Negative
Cells prior to staining are transparent.
After staining with carbolfuchsin, cells become reddish purple. No steaming is necessary.
Decolorization with acid alcohol removes stain from acid-fast negative cells.
Methylene blue is used to counterstain acid-fast negative cells.

3.104 Kinyoun Acid-Fast Stain Overview ◆ Acid-fast cells stain reddish purple; nonacid-fast cells stain blue or the color of the counterstain if a different one is used.



3.105 Acid-Fast Stain Using the Kinyoun Method ◆ This is an acid-fast stain of *Mycobacterium smegmatis* (AF+) and *Staphylococcus epidermidis* (AF−) from cultures. Again, notice the clumping of the acid-fast organisms. Brilliant green dye commonly stains more of a gray color, but it still contrasts with the carbolfuchsin of acid-fast positive cells. Some Kinyoun kits use methylene blue as the counterstain, and the preparation would look like Figure 3.103.

Members of the actinomycete genus *Nocardia* (*N. brasiliensis* and *N. asteroides* are opportunistic pathogens) are partially acid-fast. Oocysts of coccidian parasites, such as *Cryptosporidium* and *Isospora*, are also acid-fast. Because so few organisms are acid-fast, the acid-fast stain is run only when infection by an acid-fast organism is suspected.

Acid-fast stains are useful in identifying **acid-fast bacilli** (**AFB**) and in rapid, preliminary, and provisional diagnosis of tuberculosis (with greater than 90% predictive value from sputum samples). It also can be performed on patient samples to track the progress of antibiotic therapy and determine the degree of contagiousness. A prescribed number of microscopic fields are examined and the number of AFB is determined and reported using a standard scoring system.

In This Exercise

Today you will perform an acid-fast stain designed primarily to identify members of the genus *Mycobacterium*. Because you know ahead of time which organisms should give a positive result and which should give a negative result, it is okay to mix them into a single emulsion.

✓ Materials

Per Student Group

- ☐ Compound microscope with oil-immersion lens and ocular micrometer
- ☐ Clean glass microscope slides
- ☐ Staining tray
- ☐ Staining screen
- ☐ Bibulous paper or paper towel
- ☐ Slide holder
- ☐ Sterile wooden sticks or disposable plastic loops
- ☐ Ziehl-Neelsen Stains (complete kits are commercially available)
  - Methylene blue stain
  - Ziehl's carbolfuchsin stain
  - Acid alcohol (95% ethanol + 3% HCl)
- ☐ Kinyoun Stains (complete kits are commercially available)
  - Kinyoun carbolfuchsin
  - Acid alcohol (95% ethanol + 3% HCl)
  - Methylene blue stain or brilliant green stain (some kits come with this counterstain)
- ☐ Sheep serum
- ☐ Squirt bottle with water
- ☐ Heating apparatus (steam or hot plate)—for ZN only
- ☐ Nonsterile Petri dish for transporting slides
- ☐ Disposable gloves
- ☐ Chemical eye protection
- ☐ Lab coat
- ☐ Recommended organisms:
  - *Mycobacterium phlei* (BSL-2) or *M. smegmatis* (BSL-2)
  - *Staphylococcus epidermidis*

Procedure: Ziehl-Neelsen (ZN) Method

1 **Using BSL-2 precautions,** prepare a smear of each organism on a clean glass slide as illustrated in Figure 3.84, substituting a drop of sheep serum for the drop of water. Gently mix the *Mycobacterium* smear thoroughly because the cells tend to stick together in clumps (Figs. 3.103 and 3.105), but use care not to produce aerosols. Air-dry and then heat-fix the smears using a microincinerator. *Note:* Because you know which organism is acid-fast positive and which is acid-fast negative, you may make two separate smears right next to one another on the slide or mix the two organisms in one smear. This is not recommended if you are staining an unknown.

2 Follow the staining protocol shown in the procedural diagram (Fig. 3.106). Be sure to wear gloves and eye protection. Use a steaming apparatus (such as the one in Fig. 3.107) to heat the slide. If the slide must be carried to and from the steaming apparatus, put it in a covered Petri dish. ***Caution!*** Be sure to have adequate ventilation (such as a fume hood or biosafety cabinet) while staining.

3 Observe using the oil-immersion lens. Record your observations of cell morphology and arrangement, dimensions, and acid-fast reaction in the table on the data sheet, page 209.

4 When finished, dispose of slides in a disinfectant jar or sharps container.

1. Prepare a slide with up to three emulsions, each in a drop of serum (optional) Use a small inoculum and mix each smear thoroughly to separate the sticky mycobacterial cells, but do not spatter (Fig. 3.84), and then heat-fix. **Use BSL-2 precautions if appropriate.** Wearing gloves and eye protection, place the slide on the steaming apparatus (Fig. 3.107). If you need to transport the slide to another part of the lab for staining, put it in a covered Petri dish.

2. Perform this step with adequate ventilation (e.g., in a fume hood). Wearing gloves and chemical eye protection, and the slide on the steaming apparatus, cover the smear(s) with a strip of bibulous paper cut slightly smaller than the slide and apply ZN carbolfuchsin stain. Heat the slide to *steaming* for 5 minutes as shown in Figure 3.107. Keep the paper moist with stain and do not boil it.

3. Remove the bibulous paper (with forceps, if available, to keep the stain off your gloves) and dispose of it properly. Grasp the slide with a slide holder and hold it on an angle. Gently rinse both sides of the slide with distilled water into the staining tray.

4. Continue holding the slide with a slide holder. Decolorize with acid-alcohol until the runoff is clear. **Use caution when handling the acid-alcohol.**

5. Still holding the slide on an angle, gently rinse with distilled water into a staining tray.

6. If not already there, return to your lab station carrying the slide in a Petri dish. Then, place the slide on the staining tray and counterstain the smear(s) with methylene blue for one minute. Be sure the staining tray catches any excess stain.

7. Grasp the slide with a slide holder and hold it on an angle. Gently rinse the slide with distilled water into the staining tray.

8. Gently blot dry in a tablet of bibulous paper or paper towels. (Alternatively, a page from the tablet can be removed and used for blotting.) Do not rub. When dry, observe under oil immersion.

3.106 Procedural Diagram: ZN Acid-Fast Stain ♦ Use BSL-2 precautions throughout this procedure and perform the staining in a fume hood or well-ventilated area. Carry the slide to and from the steaming apparatus in a covered Petri dish.

3.107 Steaming the Slide During the Ziehl-Neelsen Procedure ◆ Carefully steam the slide to melt the waxy wall so the carbolfuchsin can get into acid-fast cells. Do not boil the slide or let it dry out. Keep it moist with stain for the entire 5 minutes of steaming. **Caution:** This must be performed in a fume hood or a biosafety cabinet with hand, clothing, and eye protection.

Procedure: Kinyoun Method

1. Using BSL-2 precautions, prepare a smear of each organism on a clean glass slide as illustrated in Figure 3.84, substituting a drop of sheep serum for the drop of water. Gently mix the *Mycobacterium* smear thoroughly because the cells tend to stick together in clumps (Figs. 3.103 and 3.105), but use care not to produce aerosols. Air-dry and then heat-fix the smears. *Note:* Because you know which organism is acid-fast positive and which is acid-fast negative, you may make two separate smears right next to one another on the slide or mix the two organisms in one smear. This is not recommended if you are staining an unknown.
2. Follow the staining protocol shown in the procedural diagram (Fig. 3.108). Be sure to wear gloves, eye protection, and perform the stain with adequate ventilation. A fume hood or a biosafety cabinet is recommended.
3. Observe using the oil-immersion lens. Record your observations of cell morphology and arrangement, dimensions, and acid-fast reaction on the data sheet.
4. When finished, dispose of slides in a disinfectant jar or a sharps container.

References

Atlas, Ronald M. and James W. Snyder. Page 278 in *Manual of Clinical Microbiology*, 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Chapin, Kimberle C. and Patrick R. Murray. Pages 259–261 in *Manual of Clinical Microbiology*, 8th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Michael A. Pfaller, and Robert H. Yolken, eds. Washington, DC: American Society for Microbiology, 2003.

Doetsch, Raymond N. and C. F. Robinow. Page 32 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Norris, J. R. and Helen Swain. Chap. II in *Methods in Microbiology*, Vol. 5A. J. R. Norris and D. W. Ribbons. eds. London, UK: Academic Press, Ltd., 1971.

Power, David A. and Peggy J. McCuen. Page 5 in *Manual of BBL™ Products and Laboratory Procedures*, 6th ed. Cockeysville, MD: Becton Dickinson Microbiology Systems, 1988.

Tille, Patricia M. Pages 73-76 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, Inc., an affiliate of Elsevier, Inc., 2014.

1. Prepare a slide with up to three emulsions, each in a drop of serum (optional). Use a small inoculum and mix each smear thoroughly to separate the sticky mycobacterial cells, but do not spatter (Fig. 3.84), and then heat-fix. **Use BSL-2 precautions if appropriate.**

2. Perform this step with adequate ventilation (e.g., in a fume hood). Wearing gloves and eye protection, place the slide on a rack over a staining tray and apply Kinyoun carbolfuchsin for 5–10 minutes. DO NOT heat the slide. Be sure the staining tray catches any excess stain.

3. Grasp the slide with a slide holder and hold it on an angle. Gently rinse the slide with distilled water into the staining tray.

4. Continue holding the slide with a slide holder. Decolorize with acid-alcohol until the run-off is clear. **Use caution when handling the acid-alcohol.**

5. Still holding the slide on an angle, gently rinse with distilled water into the staining tray.

6. Return the slide to the staining tray and counterstain the smear(s) with methylene blue or brilliant green stain for 1 minute. Be sure the staining tray catches any excess stain.

7. Grasp the slide with a slide holder and hold it on an angle. Gently rinse the slide with distilled water into the staining tray.

8. Gently blot dry in a tablet of bibulous paper or paper towels. (Alternatively, a page from the tablet can be removed and used for blotting.) Do not rub. When dry, observe under oil immersion.

3.108 Procedural Diagram: Kinyoun Acid-Fast Stain ♦ **Use BSL-2 precautions** throughout this procedure and perform the stain in a fume hood or well-ventilated area. Carry the slide to and from the fume hood in a covered Petri dish.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET

3-8

Acid-Fast Stains

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations in the table below.

Organism	Staining Method (ZN or K)	Cellular Morphology and Arrangement (include a detailed sketch of a few representative cells)	Cell Dimensions	Color	Acid-Fast Reaction (+/−)

QUESTIONS

1 *How does heating the bacterial smear during a ZN stain promote entry of carbolfuchsin into the acid-fast cell wall?*

2 *Are acid-fast negative cells stained by carbolfuchsin? If so, how can this be a differential stain?*

3 *Why do you suppose the acid-fast stain is not as widely used as the Gram stain? When is it more useful than the Gram stain?*

EXERCISE
3-9
Capsule Stain

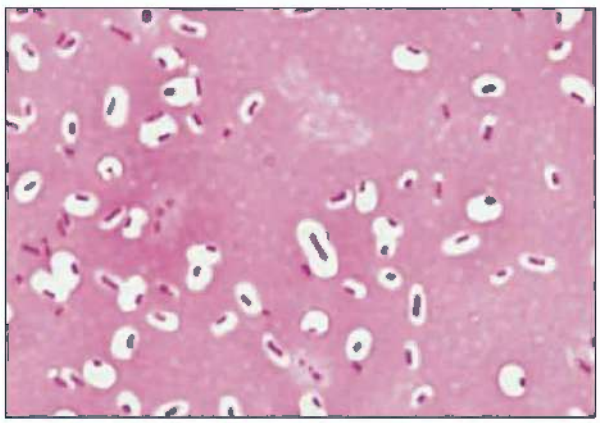
Theory

Capsules are composed of mucoid polysaccharides or polypeptides that repel most stains because of their neutral charge. The capsule stain technique takes advantage of this characteristic by staining *around* the cells. Typically, an acidic stain such as Congo red or nigrosin, which stains the background, and a basic stain that colorizes the cell proper are used in combination. The capsule remains unstained and appears as a white halo between the cells and the colored background (Fig. 3.109).

This technique begins as a negative stain; cells are spread in a film with an acidic stain and are not heat-fixed. Heat-fixing causes the cells to shrink, leaving an artifactual white halo around them that might be interpreted as a capsule. In place of heat-fixing, cells may be emulsified in a drop of serum to promote their adhering to the glass slide.

Application

The capsule stain is a differential stain used to detect cells capable of producing an extracellular capsule. Capsule production increases virulence in some microbes (such as the anthrax bacillus *Bacillus anthracis* and the pneumococcus *Streptococcus pneumoniae*) by making them less vulnerable to phagocytosis.



3.109 Capsule Stain ♦ The acidic stain colorizes the background while the basic stain colorizes the cell, leaving the capsules as unstained, white clearings around the cells. Notice the lack of uniform capsule size, and even the absence of a capsule in some cells. There are several variations of the capsule stain. The capsule stain protocol provided in this exercise will produce different colors than in this micrograph, but the principle is the same—capsules will appear as white clearings.

In This Exercise

The capsule stain allows you to visualize an extracellular capsule, if present. Be careful to distinguish between a tiny white halo (as a result of cell shrinkage) and a true capsule.

3

✓ Materials

Per Student

- ☐ Clean glass slides
- ☐ Sheep serum
- ☐ Maneval's stain
- ☐ Congo red stain
- ☐ Squirt bottle with water
- ☐ Staining tray
- ☐ Staining screen
- ☐ Coplin staining jar (or a beaker) with distilled water
- ☐ Bibulous paper or paper towel
- ☐ Slide holder
- ☐ Disposable gloves
- ☐ Chemical eye protection
- ☐ Sterile toothpicks
- ☐ Compound microscope with oil-immersion lens and ocular micrometer
- ☐ Immersion oil
- ☐ Lens paper
- ☐ Recommended organisms (18- to 24-hour skim milk or tryptic soy agar slant pure cultures)
  - *Aeromonas sobria*
  - *Rhizobium leguminosarum*
  - (Optional) *Klebsiella pneumoniae* (BSL-2)

Procedure

1. Follow the protocol in the procedural diagram (Fig. 3.110) to make stains of the organisms supplied. **Use BSL-2 precautions if *K. pneumoniae* is stained.** Use a separate slide for each specimen and do not heat-fix them. Be sure to wear gloves and eye protection.
2. Using a sterile toothpick, obtain a sample from below the gumline in your mouth. (Do not draw blood!) Mix the sample into a drop of water or serum on a slide, and then perform a capsule stain on it.

3 Observe, using the oil-immersion lens. Record your observations of cell morphology and arrangement, cell dimensions, and presence or absence of a capsule in the table on the data sheet, page 213.

4 Dispose of the specimen slides in a jar of disinfectant or sharps container after use.

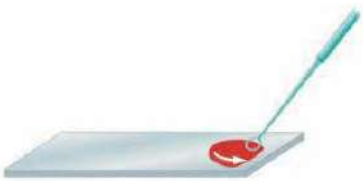
References

Hughes, Roxana B. and Ann C. Smith. "Capsule Stain Protocols," ASM MicrobeLibrary (September 29, 2007). Updated July 22, 2013. Available online. URL: http://www.microbelibrary.org/library/laboratory-test/3041-capsule-stain-protocols.

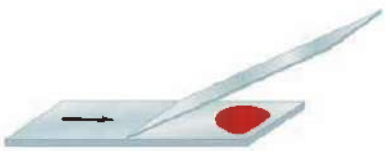
Murray, R. G. E., Raymond N. Doetsch, and C. F. Robinow. Page 35 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Norris, J. R. and Helen Swain. Chap. II in *Methods in Microbiology*, Vol. 5A. J. R. Norris and D. W. Ribbons, eds. London, UK: Academic Press, Ltd. 1971.

1. Working on the table top covered with a paper towel, place a loopful of sheep serum at one end of a clean glass slide. Add a drop of Congo red stain. Be sure to wear gloves.



2. Aseptically add organisms and emulsify with a loop. Sterilize the loop after emulsifying. ***Note:*** If you are transferring a BSL-2 organism, use a sterile wooden stick or disposable loop/needle and dispose of it properly when finished.

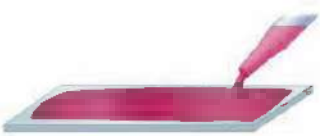


3. Take a second clean slide, place it on the surface of the first slide, and draw it back into the drop.

4. When the drop flows across the width of the spreader slide (or slightly before) ...

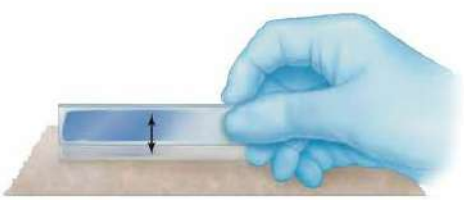
5. ... immediately push the spreader slide to the other end. Dispose of the spreader slide in a jar of disinfectant or sharps container.

6. Air-dry and do NOT heat-fix.



7. Place the slide on a rack over a staining tray. Cover the spread with Maneval's stain for 1 minute. Make sure the staining tray catches all excess stain.

8. Grasp the slide with a slide holder and dip it into a jar of distilled water two or three times to rinse off the Maneval's stain. Change the water in the jar frequently.



9. Gently tap the edge of the slide on the paper towel to remove excess water, then set it down to air-dry. When dry, observe under oil immersion.

3.110 Procedural Diagram: Capsule Stain ♦ Be sure to sterilize your loop after transfer and to appropriately dispose of the spreader slide immediately after use. Do not set it on the table. Once air-dried, the slide is ready for counterstaining. No heat-fixing is required. Gently rinsing the Maneval's stain and not blotting the slide dry will help with the quality of your preparations. Properly dispose of the slide after viewing.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-9

Capsule Stain

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations in the table below. Use a separate line for each organism from your gumline sample that illustrates a capsule or an unusual cell morphology or arrangement. Measure cell dimensions and the width of the capsule from the cell's surface to the capsule's edge.

Organism	**Cellular Morphology and Arrangement** (include a detailed sketch of a few representative cells)	**Capsule** (+/−)	**Cell Dimensions and Width of Capsule** (if any)

QUESTIONS

1 *Capsules are neutrally charged. This being the case, what is the purpose of emulsifying the sample in serum in this staining procedure?*

2 *Some oral bacteria produce an extracellular "capsule." Of what benefit is a capsule to these cells?*

3 *Sketch any cells from your mouth sample that display an unusual morphology or arrangement.*

EXERCISE
3-10

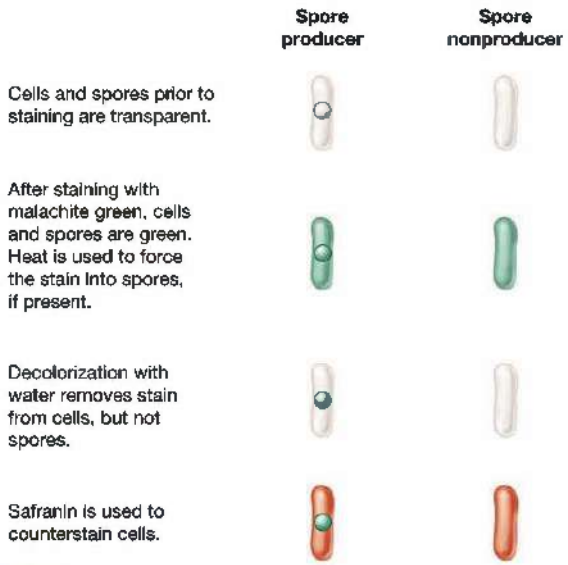
Endospore Stain

Theory

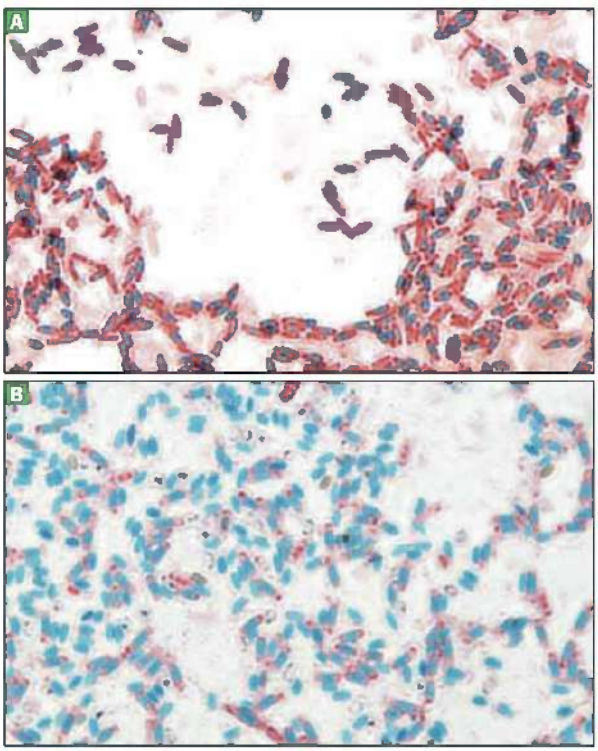
Some bacterial species are able to differentiate into dormant cells called **endospores** when environmental conditions, such as nutrient depletion or high temperatures, are unsuitable for growth. Endospores are highly resistant to heat and chemicals, which allows them to survive in this state for long periods of time. The total absence of ATP within endospores is an indication of how dormant they are! In addition to nutrient depletion, sporulation has also been shown to be dependent on population density. With increased density, a secreted peptide (called "competence and sporulation factor," or CSF) reaches a critical concentration and results in derepression of sporulation genes, which is a fancy way of saying, "Taking the brakes off" of them. Endospores' resistance is due to a combination of factors, including a tough outer covering made of the protein **keratin**, its dehydrated state, DNA protective proteins, and other adaptations. When conditions are suitable again, endospores germinate into metabolically active **vegetative cells**.

The keratin in the spore coat also resists staining, so extreme measures must be taken to stain an endospore. In the Schaeffer-Fulton method (Fig. 3.111), a primary stain of malachite green is forced into the spore by steaming the bacterial emulsion. Alternatively, malachite green can be left on the slide for 15 minutes or more to stain the spores. Malachite green is water soluble and has a low affinity for cellular material, so vegetative cells and **spore mother cells** (which are responsible for producing the endospore) can be decolorized with water and counterstained with safranin (Fig. 3.112).

Endospores may be located in the middle of the cell (**central**), at the end of the cell (**terminal**), or between the end and middle of the cell (**subterminal**). Endospore location in some species is variable and may be a combination of terminal and subterminal, for instance.

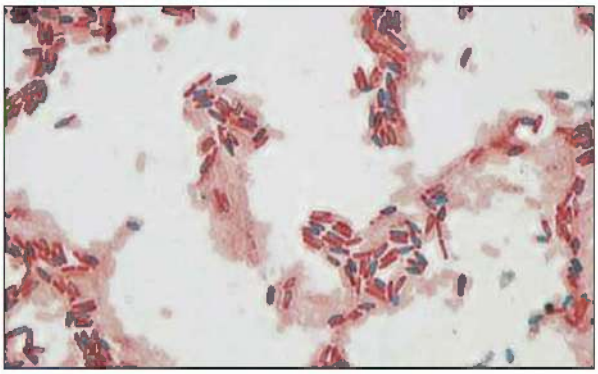

Spore producer
Spore nonproducer
Cells and spores prior to staining are transparent.
After staining with malachite green, cells and spores are green. Heat is used to force the stain into spores, if present.
Decolorization with water removes stain from cells, but not spores.
Safranin is used to counterstain cells.

3.111 The Schaeffer-Fulton Endopore Stain ♦ Upon completion, endospores are green, vegetative and spore mother cells are red.

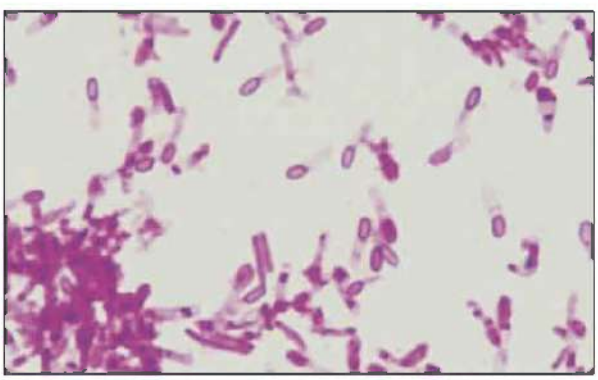


3.112 Culture Age Can Affect Sporulation ♦ Bacteria capable of producing endospores do not do so uniformly during their culture's growth. Sporulation is done in response to nutrient depletion, and so is characteristic of older cultures. These two cultures illustrate different degrees of sporulation. (A) Most cells in this specimen contain endospores; very few have been released. The light pink rods are probably spore mother cells that have released their spore and have died. (B) This specimen consists mostly of released endospores.

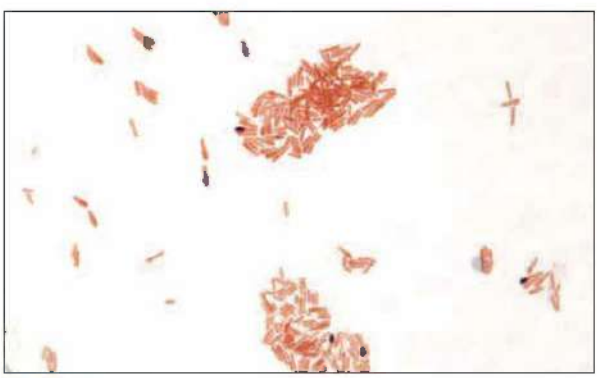
Endospores also may be differentiated based on shape—either **spherical** or **elliptical (oval)**—and size relative to the cell, that is, whether they cause the cell to look swollen or not. These structural features are shown in Figures 3.113 through 3.115.



3.113 Central Elliptical Endospores ◆ Most of these *Bacillus megaterium* endospores are centrally positioned, but some are subterminal. All are elliptical, but they do not distend the mother cell.



3.114 Terminal Elliptical Endospores ◆ Shown is *Clostridium tetani* stained by a different endospore stain protocol using carbolfuchsin. Notice how the endospores have caused the ends of the cells to distend (swell).



3.115 Subterminal Elliptical Endospores ◆ This is a stained preparation of *Clostridium botulinum* using an alternative procedure. The black, elliptical endospores slightly distend the cell.

Application

The endospore stain is a differential stain used to detect the presence, shape, and location of endospores in bacterial cells. Only a few genera produce spores. Most common are the genera *Bacillus* (over 100 species) and *Clostridium* (over 160 species). In addition, several "new" genera formerly classified as *Bacillus* species are also endospore producers. These include *Bevibacillus*, *Geobacillus*, *Paenibacillus*, and *Virgibacillus*, among others.

Most *Bacillus* species are soil, freshwater, or marine **saprophytes**, but two are pathogens. *B. anthracis* is the causal agent of anthrax and *B. cereus* causes two kinds of food poisoning: emetic and diarrheal. Most members of *Clostridium* are soil or aquatic saprophytes, or inhabitants of human intestines, but four pathogens are fairly well known: *C. tetani* (tetanus), *C. botulinum* (botulism), *C. perfringens* (gas gangrene), and *C. difficile* (pseudomembranous colitis).

In This Exercise

In the endospore stain you will examine endospores from different-aged *Bacillus* cultures. You will not have to stain each species. Divide the work within your lab group, but be sure to look at each other's slides. Also be sure to compare spore shape and location for each species.

✓ Materials

Per Student Group

- ☐ Clean glass microscope slides
- ☐ Malachite green stain
- ☐ Safranin stain
- ☐ Squirt bottle with water
- ☐ Heating apparatus (steam apparatus or hot plate)
- ☐ Fume hood
- ☐ Bibulous paper or paper towel
- ☐ Staining tray
- ☐ Staining screen
- ☐ Slide holder
- ☐ Disposable gloves
- ☐ Lab coat
- ☐ Chemical eye protection
- ☐ Compound microscope with oil-immersion lens and ocular micrometer
- ☐ Immersion oil
- ☐ Lens paper
- ☐ Nonsterile Petri dish for transporting slides

□ Recommended organisms (per student group):

- 48-hour and 5-day sporulating agar slant pure cultures of *Bacillus cereus*
- 48-hour and 5-day sporulating agar slant pure cultures of *Bacillus coagulans*
- 48-hour and 5-day sporulating agar slant pure cultures of *Bacillus megaterium*
- 48-hour and 5-day sporulating agar slant pure cultures of *Bacillus subtilis*

(*Note*: tryptic soy agar can be substituted for sporulating agar)

Medium Recipe

Sporulating Agar

• Pancreatic digest of gelatin	6.0 g
• Pancreatic digest of casein	4.0 g
• Yeast extract	3.0 g
• Beef extract	1.5 g
• Dextrose	1.0 g
• Agar	15.0 g
• Manganous sulfate	0.3 g
• Distilled or deionized water	1.0 L

Final pH 6.6 ± 0.2 at 25°C

Procedure

1. For each organism, prepare and heat-fix two smears on the same slide as illustrated in Figure 3.84. One smear should be the 48-hour culture, and the other the 5-day culture. Divide the work within your lab group. Minimally, each student should prepare one slide with smears of the 48-hour and 5-day cultures of one species. ***Note*: Use BSL-2 precautions if you have subcultured soil isolates for endospore staining.**
2. Follow the instructions in the procedural diagram in Figure 3.116. Use a steaming apparatus like the one shown in Figure 3.117. Be sure to have adequate ventilation (a fume hood is highly recommended), eye protection, and gloves. If you must carry the slide to and from the steaming apparatus, put it in a covered Petri dish.
3. Observe using the oil-immersion lens. Record your observations of cell morphology and arrangement, cell dimensions, and endospore presence, position and shape in the table provided on the data sheet, page 219.
4. Dispose of specimen slides in a disinfectant jar or a sharps container.

1. Begin with up to three heat-fixed emulsions on one slide. (Fig. 3.84.) **Use BSL-2 precautions, if appropriate.** Wearing gloves and chemical eye protection, cover the smear with a strip of bibulous paper cut slightly smaller than the slide. If you need to transport the slide to another part of the lab for staining, put it in a covered Petri dish.

2. Set the slide on a steaming apparatus and saturate the bibulous paper with malachite green stain. Heat it to steaming for 5 to 7 minutes (as shown in Figure 3.117). Be sure to keep the paper moist with stain, but don't have it so wet that stain runs off the slide. Perform this step with adequate ventilation, preferably in a fume hood.

3. Grasp the slide with a slide holder and properly dispose of the bibulous paper with forceps (to prevent stain from getting on your gloves). Then, hold the slide on an angle over a stain tray and gently rinse both sides with distilled water until the runoff is clear. Then, if not already there, return to your lab station carrying your slide in a Petri dish.

4. Place the slide on the staining rack and counterstain with safranin for one minute. Be sure excess stain falls into the staining tray.

5. Grasp the slide with a slide holder and hold it on an angle. Gently rinse the slide with distilled water into the staining tray.

6. Gently blot dry in a tablet of bibulous paper or paper towels. (Alternatively, a page from the tablet can be removed and used for blotting.) Do not rub. When dry, observe under oil immersion.

3.116 Procedural Diagram: Schaeffer-Fulton Endopore Stain ♦ Steam the staining preparation; do not boil it. Be sure to perform this procedure with adequate ventilation (preferably a fume hood), eye protection, a lab coat, and gloves. **Employ BSL-2 precautions** if you have subcultured a soil isolate for endospore staining.

3.117 Steaming the Slide During the Endospore Stain ♦
Carefully steam the slide to force the malachite green into the endospores. Do not boil the slide or let it dry out. Keep it moist with stain for up to 10 minutes of steaming (not 10 minutes on the apparatus—10 minutes of steaming!). ***Caution:*** This should be performed in a well-ventilated area (preferably a fume hood) with hand, clothing, and eye protection.

References

Claus, G. William. Chap. 9 in *Understanding Microbes—A Laboratory Textbook for Microbiology*. New York: W. H. Freeman and Co., 1989.

Logan, Niall A. and Paul De Vos. Genus I. Bacillus in *Bergey's Manual of Systematic Bacteriology*, 2nd ed., Vol. III The *Firmicutes*. Paul De Vos, George M. Garrity, Dorothy Jones, Noel R. Krieg, Wolfgang Ludwig, Fred A. Rainey, Karl-Heinz Schleifer, and William B. Whitman, eds. New York: Springer, 2009.

Murray, R. G. E., Raymond N. Doetsch, and C. F. Robinow. Page 34 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET

3-10

Endospore Stain

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations in the table below.

Organism (include culture age)	**Cellular Morphology and Arrangement** (include a detailed sketch of a few representative cells and spores)	**Cell Dimensions**	**Spores** (present or absent)	**Spore Shape, Position, and Dimensions** (if present)

QUESTIONS

1 *Why does this exercise call for an older (5-day) culture of* Bacillus*?*

3

2 *What does a positive result for the endospore stain indicate about the organism? What does a negative result for the endospore stain indicate about the organism?*

3 *Why is it not necessary to include a negative control for this stain procedure?*

4 *Endospores do not stain easily. Perhaps you have seen them as unstained white objects inside* Bacillus *species in other staining procedures. If they are visible as unstained objects in other stains, of what use is the endospore stain?*

Wet Mount and Hanging Drop Preparations

EXERCISE 3-11

Theory

A wet mount preparation is made by placing the specimen in a drop of water on a microscope slide and covering it with a cover glass. Because no stain is used and most cells are transparent, viewing is best done with as little illumination as possible by adjusting the iris diaphragm (Fig. 3.118). Motility often can be observed at low or high-dry magnification, but viewing must be done quickly because of drying of the preparation. Swimming bacteria will move independently of one another, either in a straight line or in a zigzag, random path.

A hanging drop preparation allows longer observation of the specimen because it does not dry out as quickly. A thin ring of petroleum jelly is applied around the well of a depression slide. A drop of water then is placed in the center of the cover glass and living microbes are transferred into it. A depression microscope slide is carefully placed over the cover glass in such a way that the drop is received into the depression and is undisturbed. The petroleum jelly causes the cover glass to stick to the slide.

The preparation then may be picked up, inverted so the cover glass is on top, and placed under the microscope for examination. As with the wet mount, viewing is best done with as little illumination as possible, so adjust the iris diaphragm. The petroleum jelly forms an airtight seal that slows drying of the drop, allowing a longer period for observation of cell size, shape, binary fission, and motility.

If these techniques are done to determine motility, the observer must be careful to distinguish between true motility and the **Brownian motion** created by collisions with water molecules. In the latter, cells will appear to vibrate in place. Nonmotile cells should exhibit Brownian motion. Cells that actually swim will exhibit independent movement over greater distances and the effects of collisions with water molecules will be insignificant.

In addition to Brownian motion, other complications can present themselves when observing wet mounts or hanging drop preparations for motility. Following is a list you can use to troubleshoot if you do not see motility.

- Observation: Cells are in one focal plane and not moving, even by Brownian motion. Interpretation and solution: The cells are stuck to the glass and may or may not be motile if freed. Focus on cells in the water between the coverslip and the slide.
- Observation: Cells are streaming across the field in the same direction. Interpretation and solution: There is a current carrying the cells and they may or may not be motile. The water is either receding due to evaporation or the coverslip was put on too vigorously. Try finding a calmer place to observe the cells or simply wait until the current dissipates. If the water is evaporating, make another slide.
- Observation: Cells are packed together and not moving except, perhaps, by Brownian motion. Interpretation and solution: You have got a traffic jam and the cells may or may not be motile. Look around for a part of the slide where the cells are not so densely packed. If you can't find such a region, make a new slide with fewer cells.

3.118 Wet Mount ♦ Shown is an unstained wet mount preparation of a motile Gram-negative rod. Because of the thickness of the water in the wet mount, cells show up in many different focal planes and are mostly out of focus. To get the best possible image, adjust the condenser height and reduce the light intensity with the iris diaphragm (as described in Exercise 3-1).

One last complication presents itself, and you won't know that it has occurred because most bacterial flagella are too thin to see with the light microscope. Flagella are very delicate and it is possible to damage them when transferring cells to the slide. It is best to check for motility using a broth culture. Also, do not emulsify the loopful of broth on the slide. Just gently allow the broth to merge with the water drop already on the slide.

Application

Most bacterial microscopic preparations result in death of the microorganisms as a result of heat-fixing and staining. Simple **wet mounts** and the **hanging drop technique** allow observation of living cells to determine motility. They also are used to see natural cell size, arrangement, and shape, as well as to study binary

fission. These characteristics may be useful in identification of a microbe.

In This Exercise

Today you will have the opportunity to view living bacterial cells swimming. The hanging drop preparation allows longer viewing, whereas the simple wet mount can be used to view motility, and it is the starting point for a flagella stain (Exercise 3-12).

3

✓ Materials

Per Student

- □ Compound microscope with oil-immersion lens and ocular micrometer
- □ Depression slide and cover glass
- □ Clean microscope slides and cover glasses
- □ Petroleum jelly
- □ Toothpick
- □ Immersion oil
- □ Lens paper
- □ Disposable gloves
- □ Recommended organisms (overnight cultures grown in broth media):
  - *Aeromonas sobria*
  - *Rhodospirillum rubrum*
  - *Staphylococcus epidermidis*
  - (Optional) pond water

Procedure: Wet Mount Preparation

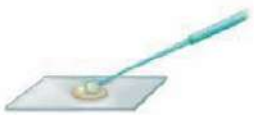
1. Place a loopful of water on a clean glass slide.
2. Gently add bacteria to the drop by placing the loop over the drop for several seconds. Do not over-inoculate and do not vigorously emulsify. Flame the loop after transfer.
3. Gently lower a cover glass with your loop supporting one side over the drop of water. Avoid trapping air bubbles.
4. Observe under high-dry power and record your results in the table on the data sheet, page 223. Avoid hitting the cover slip with the high-dry lens.
5. Dispose of the slide and cover glass in a disinfectant jar or a sharps container when finished.

Procedure: Hanging Drop Preparation

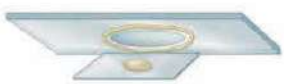
1. Follow the procedure illustrated in Figure 3.119 for each specimen.
2. Observe under high dry and record your results in the table on the data sheet. Avoid hitting the cover slip with the high-dry objective.
3. When finished, remove the cover glass from the depression slide with an inoculating loop and soak both in a disinfectant jar for at least 15 minutes. Flame the loop. After soaking, rinse the slide with 95% ethanol to remove the petroleum jelly, then with water to remove the alcohol. Dry the slide for reuse.

1. Apply a light ring of petroleum jelly around the well of a depression slide with a toothpick.

2. Apply a drop or a loopful of water to a cover glass. If using a broth culture, omit the water. Do not use too much water.



3. Aseptically, gently transfer a loopful of culture into the drop of water. (If you are transferring from a slant, get a loopful of sterile water and touch it to the growth for 15 seconds.) Then, without mixing, hold the loop in the water. Flame your loop (or dispose of it properly) after the transfer.



4. Invert the depression slide so the drop is centered in the well. Gently press until the petroleum jelly has created a seal between the slide and cover glass. Then, turn the slide right side up and place it on the stage of your microscope.

5. From the side, the preparation should look like this. Notice that the drop is "hanging," and is not in contact with the depression slide. Observe under high dry with the iris diaphragm closed down to reduce glare.

3.119 Procedural Diagram: Hanging Drop Preparation ♦

The hanging drop method is used for long-term observation of a living specimen. Gently transfer the cells into the drop, and then observe with reduced light intensity.

References

Iino, Tetsuo, and Masatoshi Enomoto. Chap. IV in *Methods in Microbiology*, Vol. 1. J. R. Norris and D. W. Ribbins, eds. London, UK: Academic Press, Ltd., 1969.

Murray, R. G. E., Raymond N. Doetsch, and C. F. Robinow. Page 26 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Quesnel, Louis B. Chap. X in *Methods in Microbiology*, Vol. 1. J. R. Norris and D. W. Ribbins, eds. London, UK: Academic Press, Ltd., 1969.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-11

Wet Mount and Hanging Drop Preparations

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations in the table below.

Organism	Procedure (wet mount or hanging drop)	Cellular Morphology and Arrangement (include a detailed sketch of a few representative cells)	Cell Dimensions	Motility (+/−)

QUESTIONS

1 *In a wet mount, each of the following complications* could *lead to a false interpretation of motility. For each, write "false positive," or "false negative," or "no effect," depending on how it* could *interfere with your reading of a motile organism and a nonmotile organism.*

Complication	Motile Organism	Motile Organism
Over-inoculation of the slide with organisms		
Cells attaching to the glass slide or cover glass		
Receding water line		
Using an old culture		

2 *You are told that viewing is best done with as little illumination as possible. Why will transparent cells be easier to view with less light?*

3

3 *Why should Brownian motion increase the longer you observe a hanging drop or wet mount preparation?*

4 *If you examined pond water, did you notice any bacteria swimming in "runs and tumbles"? Did you notice any swimming in more-or-less straight lines?*

5 *Motility because of flagella is the most common form of bacterial locomotion, but some bacteria glide. If you examined pond water, did you notice any gliding cyanobacteria?*

EXERCISE
3-12

Flagella Stain

Theory

Bacterial flagella typically are too thin to be observed with the light microscope and ordinary stains. Staining flagella is more than just adding color to them. Various special flagella stains have been developed that use a **mordant** to assist in encrusting flagella with stain to a visible thickness. Most require experience and advanced techniques, and typically are not performed in beginning microbiology classes. The method provided in this exercise, though comparatively simple, still requires practice and a bit of good luck.

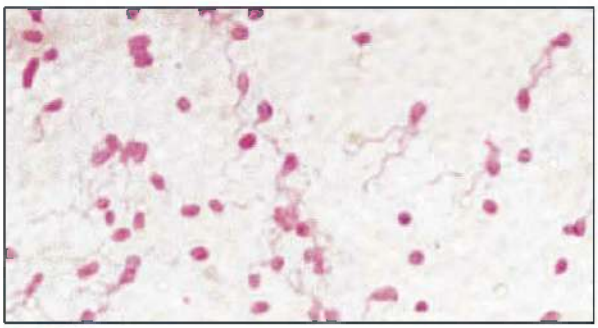
The number and arrangement of flagella may be observed with a flagella stain. A single flagellum is said to be **polar** and the cell has a **monotrichous** arrangement (Fig. 3.120). Other arrangements (shown in Figs. 3.121 through 3.123) include **amphitrichous**, with flagella at both ends of the cell; **lophotrichous**, with tufts of flagella at the end of the cell; and **peritrichous**, with flagella emerging from the entire cell surface.

3.120 Polar Flagella ◆ *Pseudomonas aeruginosa* is often suggested as a positive control for flagella stains, but it is a BSL-2 organism. Notice the single flagellum emerging from the ends of many (but not all) cells. This is a result of the fragile nature of flagella, which can be broken from the cells during slide preparation.

3.121 Amphitrichous Flagella ◆ *Spirillum volutans* has a flagellum at each end. Some spirilla are large enough that the flagella are visible with phase contrast microscopy, but they are the exceptions. As a rule, bacterial flagella must be stained to be seen with the light microscope.

Application

The flagella stain allows direct observation of flagella. The presence and arrangement of flagella may be useful in identifying bacterial species.



3.122 Lophotrichous Flagella ◆ Several flagella emerge from one end of this *Pseudomonas* species. Not all cells have flagella because they were too delicate to stay intact during the staining procedure.

3.123 Peritrichous Flagella of *Proteus vulgaris* ◆ Notice the flagella emerging from the entire cell surface. *P. vulgaris* is capable of swarming motility (Fig. 2.24), in which the cells spread across the agar surface at specific intervals, and then remain in place until the next swarm. The smaller cells are called "swimmers" and are the form seen when grown in a liquid medium. When transferred to a solid medium or under certain environmental conditions, swimmers differentiate into "swarmers." A swarmer is seen in the center of this micrograph. Swarmers are larger, contain multiple nucleoids (the site of DNA), and produce an extracellular slime or capsule that assists in swarming. After swarming, they break up into swimmer cells. *P. vulgaris* is an intestinal inhabitant of humans and animals, but can cause urinary tract infections. Swarmers are the more virulent form.

In This Exercise

You will begin this exercise with a simple wet mount preparation (Exercise 3-11). Once you have determined that the organism is motile, you will stain it to demonstrate its flagella. Be sure to note the flagellar arrangement.

✓ Materials

- ☐ Compound microscope with ocular micrometer
- ☐ Inoculating loop
- ☐ Clean microscope slides and cover slips
- ☐ Hypodermic syringe with 0.2 µm filter
- ☐ Ryu flagella stain
- ☐ Chemical eye protection
- ☐ Lab coat
- ☐ Disposable gloves
- ☐ Commercially prepared slides of motile organisms showing flagella
- ☐ Recommended organisms (overnight cultures grown in broth media):
  - *Aeromonas sobria*
  - *Rhodospirillum rubrum*
  - *Staphylococcus epidermidis*

Procedure

1. Use a separate slide for each preparation. Make sure the slide is clean, and handle it by its edges to prevent oil from your hands soiling the surface.
2. Prepare a wet mount of the organism as directed in Exercise 3-11 with the following modification. Because bacterial flagella are extremely fragile, handle the preparations gently to minimize the inevitable damage. Place a drop of water on the slide. Then place the loop over the growth and do not move it; only make contact with the growth. Allow motile bacteria to swim in for about 30 seconds. Carefully remove the loop and place it in the drop of water on the slide, again allowing motile bacteria to swim into the drop. Gently put on a cover slip.
3. Observe the specimen under high-dry power and note any motility. If the specimen is motile, continue with the procedure. If the specimen is nonmotile, observe the Brownian motion and then prepare a wet mount of a different organism.
4. If you see motility, allow the slide to dry for about 10–15 minutes. If practical to do so, leave the slide on the microscope stage so you will be able to find the cells again easily. Then apply the Ryu stain to the edge of the coverslip (Figs. 3.124 and 3.125) using the syringe. Capillary action will draw the stain under. Be careful not to get stain on the microscope.
5. Allow the preparation to stain until flagella become visible (time varies). Keep it on the microscope. Check periodically for faint, stained threads emerging from the cells. Record your observations in the table on the data sheet, page 227.
6. Dispose of your specimen slide in a disinfectant jar or sharps container after use.
7. Examine prepared slides of motile organisms and record your results on the data sheet.

3.124 Syringe with Ryu Stain ♦ Apply the stain using a syringe fitted with a 0.2 µm filter. For safety, we strongly recommend not using a needle with the syringe, but care must be taken not to deliver too much stain.

3.125 Applying the Stain ♦ Carefully place a couple of drops of stain next to the coverslip. Capillary action will draw the stain under. After about 10 minutes of staining, begin examining the slide for flagella. Be careful not to spill stain on the microscope or get it on top of the cover slip where it could get transferred to the objective lens. Clean up any spills thoroughly.

References

Heimbrook, Margaret E., Wen Lan L. Wang, and Gail Campbell. "Staining Bacterial Flagella Easily." *J. Clin. Microbiol* 27 (1989): 2612–2615.

Iino, Tetsuo and Masatoshi Enomoto. Chap. IV in *Methods in Microbiology*, Vol. 5A. J. R. Norris and D. W. Ribbins, eds. London, UK: Academic Press, Ltd, 1971.

Murray, R. G. E., Raymond N. Doetsch, and C. F. Robinow. Page 35 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology,1994.

Penner, John L. Genus XXIX. Proteus in *Bergey's Manual of Determinative Bacteriology*, 2nd ed. Vol. Two, The *Proteobacteria*, Part B, The *Gammaproteobacteria*. Don J. Brenner, Noel R. Krieg, and James T. Stanley, eds. New York: Springer, 2005.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 3-12

Flagella Stain

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations in the table below.

Organism	Cellular Morphology and Arrangement (include a detailed sketch of a few representative organisms with their flagella)	Cell Dimensions	Flagellar Length

QUESTIONS

1 *Why can't flagella be observed in action?*

2 *Flagella have a diameter of about 1 nm. A hypothetical question: To resolve flagella, what is the maximum wavelength of the electromagnetic spectrum that would have to be used to create the image (given numerical apertures of 1.25 for both the condenser and the oil lens)? Refer to Exercise 3-1 for help with this question.*

3 *Why are cells allowed to swim into and out of the loop's water film rather than scraping the agar surface of growth and then emulsifying cells on the slide?*

4 *Based on the samples of motile bacteria you have observed in Exercise 3-11 and this exercise, what general conclusion can you draw about cell morphology and the possession of flagella?*

Morphological Unknown

EXERCISE 3-13

Theory

In this exercise you will be given one pure bacterial culture selected from the organisms listed in Table 3-5. Your job will be to identify it using only the staining techniques covered in Section 3.

Your first task is to convert the information organized in Table 3-5 into flowchart form. A flowchart is simply a visual tool to illustrate the process of elimination that is the foundation of unknown identification. We have started it for you on the data sheet, page 233, by giving you a few of the branches and listing appropriate organisms. You must complete it by adding necessary branches until you have shown a path to identify each of the organisms. The following Procedure contains a detailed explanation of the process.

Once you have designed your flowchart, you will run one stain at a time on your organism. As you match your staining results with those in the flowchart, you will follow a path to its identification; each staining result will inform you of the next stain to run.

A final stain will be performed as a **confirmatory test.** It will serve as further evidence that you have identified your unknown correctly. The only hard and fast rule about confirmatory tests is that they can't have been used during the identification process. Beyond that, it is *nice* if the confirmatory test also serves to differentiate the identified organism from the other organisms on the last branch of the flowchart but that is not a requirement. It is also *nice* if the confirmatory test is anticipated to give a positive result because we generally have more confidence in positive results than negative results. However, in this exercise you do not have many stains to choose from as confirmatory tests, so do your best. Another consideration is that you might want to choose a particular stain for the confirmatory test simply for practice because it was difficult for you.

Application

Schemes employing differential tests are the main strategy for microbial identification.

In This Exercise

You will practice the stains you have learned to this point, as well as familiarize yourself with the standard process of elimination used in bacterial identification.

✓ Materials

Per Class

- ☐ Unknown organisms in numbered tubes (one per student). Fresh broth or slant cultures of[1]
  - *Bacillus subtilis*
  - *Corynebacterium xerosis*
  - *Lactococcus lactis*
  - *Micrococcus luteus*
  - *Mycobacterium phlei* (BSL-2) or *Mycobacterium smegmatis* (BSL-2)
  - *Neisseria sicca*
  - *Pseudomonas putida*
  - *Rhizobium leguminosarum*
  - *Rhodospirillum rubrum*
  - *Shigella flexneri* (BSL-2)
  - *Staphylococcus epidermidis*

(***Note:*** Your instructor will choose organisms appropriate to your specific microbiology course and facilities.)

- ☐ Sterile tryptic soy broth tubes (for subculturing)
- ☐ Sterile tryptic soy agar slants (for subculturing)
- ☐ (Optional) sporulating agar

Per Student Group

- ☐ 24-hour tryptic soy agar slant cultures in tubes labeled with Gram reaction to be used as controls
- ☐ 24-hour tryptic soy agar slant culture of an acid-fast positive organism to be used as a control (BSL-2)
- ☐ Gram stain kit
- ☐ Acid-fast stain kit
- ☐ Capsule stain kit
- ☐ Spore stain kit

Per Student

- ☐ Compound microscope with oil-immersion lens
- ☐ Immersion oil
- ☐ Clean microscope slides
- ☐ Clean cover glasses
- ☐ Lens paper
- ☐ Bunsen burner
- ☐ Striker
- ☐ Disposable inoculating loops or sterile wooden sticks

[1] Cultures should have abundant growth.

TABLE 3-5 Table of Results for Organisms Used in the Exercise ♦ These results are typical for the species listed. Your specific strains may vary because of their genetics, their age, or the environment in which they are grown. It is important that you record your results in case strain variability leads to misidentification. Also, pay attention to the footnotes!

Table of Results							
Organism	Gram Stain	Cell Morphology	Cell Arrangement[1]	Acid-Fast Stain	Motility (wet mount)[2]	Capsule[3]	Spore Stain[4]
Bacillus subtilis	+	rod	usually single cells	–	+	±	+
Corynebacterium xerosis	+	irregular rods	single cells or multiples in angular or palisade arrangement	–	–	–	–
Lactococcus lactis	+	ovoid cocci (appearing stretched in the chain or pair)	chains and some pairs	–	–	+	–
Micrococcus luteus	+	cocci	tetrads (some singles and pairs)	–	–	–	–
Mycobacterium phlei (BSL-2) or *Mycobacterium smegmatis* (BSL-2)	weak +	thin rods	single cells or branched; sometimes in dense clusters	+	–	–	–
Neisseria sicca	–	cocci	pairs and/or tetrads with adjacent sides flattened	–	–	+	–
Pseudomonas putida	–	straight to slightly curved rods	single cells	–	+	–	–
Rhizobium leguminosarum	–	short rods	usually single rods	–	–	+	–
Rhodospirillum rubrum	–	thick spirilla or bent rods	single	–	+	–	–
Shigella flexneri (BSL-2)	–	rods	single	–	–	–	–
Staphylococcus epidermidis	+	cocci	clusters and some singles, pairs, and tetrads	–	–	–	–

[1] It may be necessary to grow your unknown in broth culture to see the arrangement clearly. Because the purpose of this lab is to practice the stains, use cell arrangement in the identification flowchart only as a last resort.

[2] You might have better luck looking for motility if you transfer some culture to a small amount of sterile broth (in a microtube, for instance) and incubate it for an hour or so at 35°C. Then, make your wet mount from that culture.

[3] For purposes of this exercise, to be considered a capsule, the white halo needs to be at least the thickness of the cell. If you suspect cells have shrunk, measure them and compare their size and the size of the "capsule" to the cell dimensions as measured in your Gram stain.

[4] If spores are not readily visible, continue incubating your culture for another 24–48 hours and check again. You may also inoculate sporulation agar and incubate for 24–48 hours.

Procedure

1. Using the information contained in Table 3-5, complete the flowchart on the data sheet, page 233. Each of the 11 organisms should occupy a solitary position at the end of a branch. Do not include stains in a branch that do not differentiate any organisms. Also, be sure the names and number of organisms on any level are equal to the names and number of organisms leading into that branch. Have your instructor check your flowchart before you begin staining.
2. Obtain one unknown slant or broth culture. Record its number in the space labeled "Unknown number" in step 2 on the data sheet.
3. *NOTE:* **Because some of the unknowns are BSL-2 organisms, all procedures must be done with BSL-2 precautions.**
4. Perform a Gram stain of the unknown organism. The stain should be run with known Gram-positive and Gram-negative controls to verify your technique (Fig. 3.94). In addition to providing information on Gram reaction, the Gram stain will allow observation of cell size, shape, and arrangement. Enter all these and the date in the appropriate boxes of the table provided on the data sheet. (*Note:* Cell size for the unknown candidates is not given and should not be used to differentiate the species. To give you an idea of typical cell sizes, the cocci should be about 1 µm in diameter, and the rods 1 µm to 5 µm in length and about 1 µm in width.)
5. If you have cocci, or are unclear about cell arrangement from the Gram stain, aseptically transfer your unknown to sterile tryptic soy broth and examine it again in 24–48 hours. Cell arrangement often is easier to interpret using cells grown in broth.
6. Based on the Gram stain and cell morphology results, follow the appropriate branches in the flowchart until you reach the list of remaining organisms that matches (and hopefully, includes!) your unknown.
7. Perform the next stain and determine to which new branch of the flowchart your unknown belongs. Record the result and date of this stain in the table provided on the data sheet.
8. Repeat step 7 until you eliminate all but one organism in the flowchart—this is your unknown! (If you are not doing all the stains on the day the unknowns were handed out, be sure to inoculate appropriate media so you will have fresh cultures to work with. If you need to do an endospore stain, incubate your original culture for another 24–48 hours or inoculate sporulation agar and incubate it for 24–48 hours, and then perform the stain.) If checking for motility, inoculate a broth and incubate it for 24–48 hours.
9. After you identify your unknown, perform one more stain to confirm your result. This confirmatory test must not be one you used in the identification process and should be added to your flowchart. The result of this test should agree with the predicted result (as given in Table 3-5). If your confirmatory test result does not match the expected result, repeat any suspect stains and find the source of error. (*Note:* The source of error may be your technique or bacterial strain variability. The results in Table 3-5 are typical for each organism, but some strains may vary. By rerunning suspect stain(s) *with controls*, if appropriate, you probably will be able to identify the source of error.)
10. Use a colored marker to highlight the path on the flowchart that leads to your unknown. Have your instructor check your work.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET

3-13

Morphological Unknown

OBSERVATIONS AND INTERPRETATIONS

1 *Complete the flowchart using the information in Table 3-5, page 230. Each path should end with a single organism.*

Micrococcus luteus
Lactococcus lactis
Staphylococcus epidermidis
Bacillus subtilis
Corynebacterium xerosis
Mycobacterium smegmatis (BSL-2)

Pseudomonas putida
Rhizobium leguminosarum
Shigella flexneri (BSL-2)
Neisseria sicca
Rhodospirillum rubrum

Gram stain

+

Micrococcus luteus
Lactococcus lactis
Staphylococcus epidermidis
Bacillus subtilis
Corynebacterium xerosis
Mycobacterium smegmatis (BSL-2)

Cell Morphology

rod

Bacillus subtilis
Corynebacterium xerosis
Mycobacterium smegmatis (BSL-2)

coccus

Micrococcus luteus
Lactococcus lactis
Staphylococcus epidermidis

–

Pseudomonas putida
Rhizobium leguminosarum
Shigella flexneri (BSL-2)
Neisseria sicca
Rhodospirillum rubrum

Cell Morphology

coccus

Neisseria sicca

spiral

Rhodospirillum rubrum

rod

Pseudomonas putida
Rhizobium leguminosarum
Shigella flexneri (BSL-2)

2 *Record your stain results and the date each was run in the table below. Include sketches (as appropriate) along with your written descriptions. Sketch the cells carefully—do not scribble a bunch of dots or squiggles.*

Unknown number: _____

	Gram Reaction **Cell Morphology and Arrangement** **Cell Dimensions**	**Acid-Fast Stain**	**Motility** (wet mount)	**Capsule Stain**	**Spore Stain** (shape and location)
Date(s) Run					
Results	Gram Reaction: Cell Morphology and Arrangement Cell Dimensions: Sketch			Sketch:	Sketch:

3 *Match your results with those in the flowchart. Highlight the path on the flowchart that leads to identification of your unknown.*

4 *Highlight your confirmatory test in the results table.*

5 *Write the identity of your unknown organism in the space below.*

My unkown is: _____

SECTION 4

Selective Media

Individual microbial species in a **mixed culture** must be isolated and cultivated as **pure cultures** before they can be tested and properly identified. The most common means of isolating an organism from a mixed culture is to streak for isolation. Refer to Exercise 1-4 for a description of the streak-plate isolation method.

The **media** introduced in this section are designed to enhance the isolation procedure by inhibiting growth of some organisms while encouraging the growth of others. Thus, they are referred to as **selective media**. Many types of selective media contain indicators to expose differences between organisms, which also make them **differential media**. The media illustrated in this section are used specifically to isolate pathogenic Gram-negative bacilli and Gram-positive cocci from human or environmental samples containing a mixture of microorganisms.

Clinical microbiologists, who are familiar with human pathogens and the types of infections they cause, choose selective media that will screen out normal flora that also are likely to be in a sample. Environmental microbiologists often choose selective and differential media to detect **coliform** bacteria. Coliforms are a subgroup of *Enterobacteriaceae* (*Escherichia coli* being the most prominent member) that produce acid and gas from lactose fermentation. Fermentation will be discussed more fully in Section 5. Most coliforms are normal inhabitants of the human intestinal tract; therefore, their presence in the environment may be evidence of fecal contamination.

In the exercises that follow, you will examine some commonly used selective media for isolating Gram-positive cocci and Gram-negative rods. Most of the exercises contain two examples of growth—spot inoculations with pure cultures to magnify growth characteristics and streak plates of mixed samples to illustrate actual colony morphology.

All of the exercises contain a Table of Results, identifying the various reactions produced on a specific medium. These include color results, interpretations of results, symbols used to quickly identify the various reactions, and presumptive identification of typical organisms encountered with these media. Presumptive identification is not final identification. It is an "educated guess" based on evidence provided by the selective/differential medium coupled with information about the origin of a sample.

A Word about Selective Media

As you read the Theory of each of the following media and learn a little about how the tests actually perform on a biochemical level, you will see that each medium has six fundamental features. For a fuller understanding of the media and tests included in this section, watch for these six features and make sure you can identify them. (***Note:*** In the following, a distinction is made between nutritional components and substrates. Since, in the strictest sense all reactants in a biochemical reaction are substrates, this is an artificial distinction. We separate the terms to illustrate the importance of the specific substance added that allows observations of biochemical distinctions to be made.)

1. Selective, Differential, Defined, or Undefined—Which Is It?

The medium may be any combination of the above. If it is selective, it encourages growth of some organisms and discourages (inhibits) growth of others. If it is differential, it allows us to distinguish between different microbes. If it is defined, or "chemically defined," each of its ingredients is known and in exactly what amounts. If it is undefined or "complex," it contains one or more ingredient(s) of unknown composition and/or amount, such as yeast extract, beef extract, digest of gelatin, etc.

2. Nutritional Components

Nutritional components are selected to obtain the optimum growth of the organisms being tested for or suspected of being in the sample. Although many ingredients are suitable for many different types of microorganisms, typically, slight variations are made to tailor the medium for a specified group. For instance, yeast extract can be added for fastidious organisms requiring specific vitamins.

3. Inhibitors

Inhibitors make the medium selective. They are designed to exploit weaknesses in specific groups of organisms and thus prevent or inhibit their growth, while allowing other organisms to grow. Some inhibitors function by interrupting DNA synthesis or expression of a gene. Other inhibitors function at the enzymatic level, interfering with a critical reaction. Still other inhibitors interfere with membrane permeability, thus upsetting homeostasis and starting a cascade of catastrophic changes inside the cell. Sodium chloride can be added to inhibit organisms that can't tolerate high osmotic pressures. The precise "menu" of nutritional components can enhance selectivity by excluding certain ingredients required by undesired organisms in the sample. (In the absence of an inhibitor, this strategy would make it an enrichment medium.)

4. Substrates

Substrates are almost always what make the medium differential. Differentiation and identification of organisms frequently relies on their differing abilities to perform a specific chemical reaction or set of reactions and to do them in a way that can be observed. This can all be staged in an artificial environment (differential medium) by providing the organisms all the required components for growth and by including at least one substrate that only one organism or group of organisms can utilize. Once the reaction has taken place, presumably, organisms that before the test were indistinguishable are now (with the help of indicators—see #5) easily differentiated. For example, if a fecal sample is being tested for the presence of a pathogen known not to ferment lactose, then lactose is an important substrate needed to identify and isolate *E. coli* and other coliforms, all of which ferment lactose and all of which are likely to be found in the sample. Colonies of lactose non-fermenters will be visibly different and would be grown in pure culture for further testing.

5. Indicators

Indicators make a desired or expected reaction visible. An indicator is virtually always included in differential media because bacterial growth, by itself, is usually not enough to reliably differentiate between microbial groups. The medium must change fairly dramatically for us to be able to see it. Subtle changes can lead to false readings. The indicator frequently is a dye that changes color as pH changes. All dyes have a specific functional range and are chosen based on the starting pH of the medium, which in turn is based on the optimum pH of the organisms being tested.

Indicators can also be chemicals that react with products of a reaction and produce a color change. For example, if the organism being tested reduces the medium substrate, an indicator that is oxidized (ferric sulfate in solution releases Fe^{3+}) can produce a pretty spectacular change. Oxidized iron is a common reactant used to form a visible precipitate.

6. Positive or Negative?

The last item covered is a description of the positive and negative reactions. We tell you what to look for. ◆

Selective Media for Isolation of Gram-Positive Cocci

Gram-negative organisms frequently inhibit growth of Gram-positive organisms when they are cultivated together. Therefore, when looking for staphylococci or streptococci in a clinical sample, it may be necessary to begin by streaking the unknown mixture onto a selective medium that inhibits Gram-negative growth. The four selective media introduced in the first part of Section 4—Phenylethyl Alcohol Agar, Columbia CNA Agar, Bile Esculin Agar, and Mannitol Salt Agar—are all used to isolate streptococci, enterococci, or staphylococci in human samples. ◆

EXERCISE 4-1

4

Phenylethyl Alcohol Agar

Theory

Phenylethyl alcohol agar (PEA) is an undefined, selective medium that inhibits the growth of many Gram-negative and some Gram-positive organisms. It is not a differential medium because no distinction is made between organisms that successfully grow on it. Digests of casein and soybean meal provide nutrition while sodium chloride provides a stable osmotic environment suitable for the addition of sheep blood if desired. Phenylethyl alcohol is the selective agent and may be bacteriostatic or bactericidal, depending on concentration. The major effect on susceptible bacteria is physical disruption of the cytoplasmic membrane, which affects its ability to act as a permeability barrier. One noteworthy consequence of this is a rapid loss of potassium ions by the cell, which in turn affects the cell's ability to maintain pH and osmotic homeostasis. A second effect is inhibition of DNA synthesis, which is likely due to loss of molecules essential for this process.

Application

PEA is used to isolate staphylococci and streptococci (including enterococci and lactococci) from specimens containing mixtures of bacterial flora. Typically, it is used to screen out the common contaminants *Escherichia coli* and *Proteus* species. When prepared with 5% sheep blood, it is used for cultivation of Gram-positive anaerobes.

In This Exercise

You will spot-inoculate one PEA plate and one nutrient agar (NA) plate with three test organisms. (In a clinical application, PEA plates would be streaked for isolation with a patient or environmental sample.) The NA plate will serve as a comparison for growth quality on the PEA plate.

✓ Materials

Per Student Group

- ☐ One PEA plate
- ☐ One NA plate
- ☐ Fresh broth cultures of:
  - *Escherichia coli*
  - *Staphylococcus epidermidis*
  - *Streptococcus gallolyticus*

Medium Recipes

Phenylethyl Alcohol Agar

• Pancreatic digest of casein	15.0 g
• Papaic digest of soybean meal	5.0 g
• Sodium chloride	5.0 g
• β-Phenylethyl alcohol	2.5 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L
pH 7.1–7.5 at 25°C	

Nutrient Agar

• Beef extract	3.0 g
• Peptone	5.0 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L
pH 6.6 – 7.0 at 25°C	

Procedure

Lab One

1. Gently mix each culture according to your lab's standards.
2. Using a permanent marker, divide the bottom of each plate into three sectors.
3. Label the plates with the organisms' names, your name, and the date.
4. Spot inoculate (refer to Appendix B) the sectors on the PEA plate with the test organisms.
5. Repeat step 4 with the NA plate. Use the same positions for each specimen as used on the PEA plate.
6. Invert and incubate the plate at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Examine and compare the plates for quality of growth (no growth, poor growth, or good growth). See Figure 4.1 for an example.
2. Record your results on the data sheet, page 239. Refer to Table 4-1 as needed.

References

Atlas, Ronald M. and James W. Snyder. Page 297 in *Manual of Clinical Microbiology*, 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Corre, J, J. J. Lucchini, G. M. Mercier, and A. Cremieux. "Antibacterial activity of phenethyl alcohol and resulting membrane alterations." *Res Microbiol* 141, no. 4 (1990): 483–497.

Forbes, Betty A., Daniel F. Sahm, and Alice S. Weissfeld. Chap. 10 in *Bailey & Scott's Diagnostic Microbiology*, 11th ed. St. Louis, MO: Mosby, 2002.

Silva, M. T., J. C. Sousa, M. A. Macedo, J. Polónia, and A. M Parente. "Effects of phenylethyl alcohol on Bacillus and Streptococcus." *J. Bacteriol* 127, no. 3 (1976): 1359–1369.

Silver, S. and L. Wendt. "Mechanism of Action of Phenethyl Alcohol: Breakdown of the Cellular Permeability Barrier." *J. Bacteriol* 93, no. 2 (1967): 560–566.

Tille, Patricia M. Page 236 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power. Page 443 in *Difco™ & BBL™ Manual–Manual of Microbiological Culture Media*. Sparks, MD: Becton, Dickinson and Co., 2003.

4.1 Brain-Heart Infusion Agar (BHIA) versus Phenylethylalcohol Agar (PEA) ◆ PEA, a medium selective for Gram-positive organisms, is on the left and BHIA, a nutritious general growth medium, is on the right. Both plates were inoculated with the same four organisms in the same relative positions on the plates: two Gram-positive cocci and two Gram-negative rods. All grow well on BHIA, but only the Gram positives grow well on PEA. Notice that the Gram negatives demonstrate stunted growth and are not completely inhibited by PEA.

TABLE 4-1 **PEA Results and Interpretations**

Result	Interpretation	Presumptive ID
Poor growth (P) or no growth (N)	Organism is inhibited by phenylethyl alcohol	Probable Gram-negative organism
Good growth (G)	Organism is not inhibited by phenylethyl alcohol	Probable *Staphylococcus, Streptococcus, Enterococcus,* or *Lactococcus*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 4-1

Phenylethyl Alcohol Agar

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 4-1, page 238, when recording your results and interpretations in the table below.

Organism	Growth (N/P/G)		Interpretation and Presumptive ID
	PEA	NA	

QUESTIONS

1 *In your own words, what is the application (purpose) of PEA?*

2 *Which ingredient(s) in PEA supply(ies)*

a. *Carbon?*

b. *Nitrogen?*

3 *Is PEA a defined or an undefined medium? Provide the reasoning behind your choice and explain why this formulation is desirable.*

4 *In your own words, what is the role of β-phenylethyl alcohol in PEA and how does it work?*

5 *Think about the results you recorded.*

a. *Growth on the PEA and NA plates was recorded as "good growth," "poor growth," or "no growth." These are qualitative and, at least for the first two, subjective terms. What did you use to establish what constituted "good growth?"*

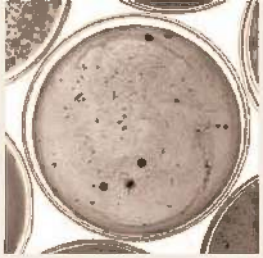
b. *Why wouldn't it be advisable to compare growth of the organisms on each plate to each other? There are at least two answers to this question!*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 4-1

(continued)

6 *Would removing β-phenylethyl alcohol from PEA alter the medium's sensitivity or specificity? (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

7 *If you observed growth of Gram-negative organisms on the PEA plate you inoculated, does this negate the usefulness of PEA as a selective medium? Why or why not?* (**Hint:** *Compare the growth on the NA and PEA plates.*)

8 *PEA contains only 0.25% phenylethyl alcohol because high concentrations inhibit both Gram-positive and Gram-negative organisms. Describe some possible reasons why this occurs.*

Columbia CNA with 5% Sheep Blood Agar

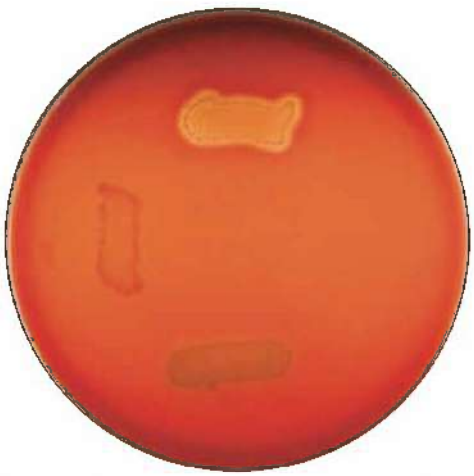
EXERCISE 4-2

Theory

Columbia CNA with 5% sheep blood agar is an undefined, differential, and selective medium that allows growth of Gram-positive organisms (especially staphylococci, streptococci, and enterococci) and stops or inhibits growth of most Gram-negative organisms (Fig. 4.2). Casein, digest of animal tissue, beef extract, yeast extract, corn starch, and sheep blood provide a range of carbon and energy sources to support a wide variety of organisms. In addition, sheep blood supplies the X factor (heme) and yeast extract provides B-vitamins. The antibiotics colistin and nalidixic acid (CNA) act as selective agents against Gram-negative organisms by affecting membrane integrity and interfering with DNA replication, respectively. They are particularly effective against *Klebsiella, Proteus,* and *Pseudomonas* species. Further, sheep blood makes possible differentiation of Gram-positive organisms based on hemolytic reaction (see Exercise 5-25).

Application

Columbia CNA agar is used to isolate staphylococci, streptococci, and enterococci, primarily from clinical specimens. The inclusion of 5% sheep blood allows differentiation based on hemolysis reaction.



4.2 Columbia CNA with 5% Sheep Blood Agar ♦ This medium is selective for Gram positives and also allows determination of hemolytic reaction (Exercise 5-25). Shown is a plate inoculated with four organisms: three Gram-positive cocci and one Gram-negative rod. Only the Gram-positive organisms grew well on the Columbia CNA agar. The Gram negative at the right didn't grow. Further, the top Gram-positive coccus is β-hemolytic, whereas the lower one is α-hemolytic and the one on the left is nonhemolytic.

In This Exercise

You will spot inoculate one Columbia CNA with 5% sheep blood agar plate and one nutrient agar (NA) plate with four test organisms. (In a clinical application, CNA plates would be streaked for isolation with a patient sample.) The NA plate will serve as a comparison for growth quality on the Columbia CNA with 5% sheep blood agar plate.

4

✓ Materials

Per Student Group

- ☐ One Columbia CNA with 5% sheep blood agar plate[1]
- ☐ One nutrient agar plate
- ☐ Fresh broth cultures of:
  - *Enterococcus faecalis* (BSL-2)
  - *Escherichia coli* (or other Gram-negative, as available)
  - *Staphylococcus epidermidis*
  - *Streptococcus gallolyticus*
  - A β-hemolytic streptococcus may be added if desired (BSL-2)

Medium Recipes

Columbia CNA with 5% Sheep Blood Agar

• Pancreatic digest of casein	12.0 g
• Peptic digest of animal tissue	5.0 g
• Yeast extract	3.0 g
• Beef extract	3.0 g
• Corn starch	1.0 g
• Sodium chloride	5.0 g
• Colistin	10.0 mg
• Nalidixic acid	10.0 mg
• Agar	13.5 g
• Distilled or deionized water *pH 7.1–7.5 at 25°C*	1 L
• Sheep blood (defibrinated)	50 mL added to 950 mL of above

[1]Columbia CNA agar without sheep blood may be substituted, but the ability to differentiate organisms based on hemolysis will be sacrificed.

Nutrient Agar

• Beef extract	3.0 g
• Peptone	5.0 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L
pH 6.6–7.0 at 25°C	

Procedure

Lab One

1. Gently mix each culture according to your lab's standards.
2. Using a permanent marker, divide the bottom of each plate into four sectors (five if a β-hemolytic streptococcus is added).
3. Label the plates with the organisms' names, your name, and the date.
4. Spot inoculate (refer to Appendix B) the sectors on the CNA plate with the test organisms. **Use BSL-2 precautions when transferring *E. faecalis* and β-hemolytic streptococcus, if used.**
5. Repeat step 4 with the nutrient agar plate. Use the same positions for each specimen as used on the CNA plate.
6. Invert and incubate the plate at 35 ± 2°C for 24 to 48 hours. If possible, incubate the plates in a candle jar or anaerobic jar with 3%–5% CO_2.

Lab Two

1. Referring to Table 4-2, examine and compare the plates for quality of growth (none, poor, or good) and hemolytic reaction. For more information on hemolysis, see Exercise 5-25.
2. Record your results in the space provided on the data sheet, page 245.

References

Chapin, Kimberle C. and Tsai-Ling Lauderdale. Chap. 21 in *Manual of Clinical Microbiology,* 9th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Marie Louise Landry, and Michael A. Pfaller, eds. Washington, DC: ASM Press, 2007.

Tille, Patricia M. Pages 86 and 236 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power. Page 156 in *Difco™ & BBL™ Manual–Manual of Microbiological Culture Media.* Sparks, MD: Becton, Dickinson and Co., 2003.

TABLE 4-2 Columbia CNA with 5% Sheep Blood Agar Results and Interpretations

Table of Results		
Result	**Interpretation**	**Presumptive ID**
Poor growth (P) or no growth (N)	Organism is inhibited by colistin and nalidixic acid	Probable Gram-negative organism
Good growth (G) with clearing of medium (β-hemolysis)	Organism is not inhibited by colistin and nalidixic acid and completely hemolyzes RBCs	Probable β-hemolytic *Staphylococcus, Streptococcus,* or rarely *Enterococcus* (***Note:*** Artifactual greening may occur with some β-hemolytic organisms
Good growth (G) with greening of medium (α-hemolysis)	Organism is not inhibited by colistin and nalidixic acid and partially hemolyzes RBCs	Probable α-hemolytic *Staphylococcus, Streptococcus,* or *Enterococcus*
Good growth (G) with no change of medium's color (γ-hemolysis)	Organism is not inhibited by colistin and nalidixic acid and does not hemolyze RBCs	Probable γ-hemolytic *Staphylococcus, Streptococcus,* or *Enterococcus*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 4-2

Columbia CNA with 5% Sheep Blood Agar

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 4-2, page 244, when recording your results and interpretations in the table below.

Organism	Growth (N/P/G)		Hemolysis (α,β,γ)	Interpretation and Presumptive ID
	Columbia CNA Agar	NA		

QUESTIONS

1 *In your own words, what is the application (purpose) of Columbia CNA with 5% sheep blood agar?*

2 *Which ingredient(s) in Columbia CNA plus 5% sheep blood agar supply(ies)*

a. *Carbon?*

b. *Nitrogen?*

3 *Is Columbia CNA agar a defined or an undefined medium? Provide the reasoning behind your choice and explain why this formulation is desirable.*

4

4 *In your own words, what are the roles of colistin and nalidixic acid in CNA and how does each work?*

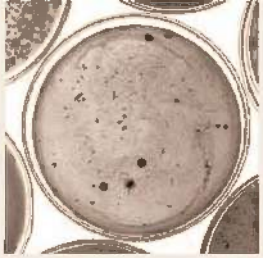
5 *In your own words, what is the role of sheep blood in CNA agar?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 4-2

(continued)

6 *Think about the results you recorded.*

a. *Growth on the Columbia CNA and NA plates was recorded as "good growth," "poor growth," or "no growth." These are qualitative and, at least for the first two, subjective terms. What did you use to establish what constituted "good growth?"*

b. *Why wouldn't it be advisable to compare growth of the organisms on each plate to each other? There are at least two answers to this question!*

7 *Would removing colistin and nalidixic acid from CNA alter the medium's sensitivity or specificity? (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

8 *Why might colistin affect Gram-negative bacteria more severely than Gram-positive bacteria?* (**Hint:** *Consider their structural differences.*)

4

9 *Compare the recipes of nutrient agar and Columbia CNA agar. If an organism can grow on both media, on which would you expect it to grow better? Explain your answer.*

EXERCISE
4-3
Bile Esculin Agar

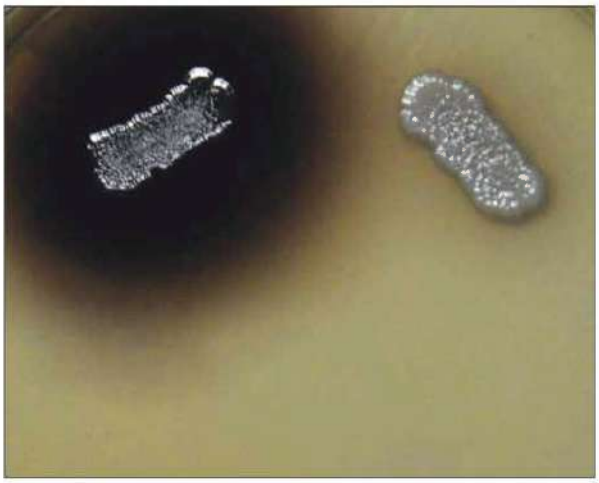
Theory

Bile esculin agar (BEA) is an undefined, selective, and differential medium containing beef extract, digest of gelatin, esculin, oxgall (bile), and ferric citrate. Esculin extracted from the bark of the horse chestnut tree is a glycoside composed of glucose and esculetin. Beef extract and gelatin provide nutrients and energy; bile (oxgall) is the selective agent added to separate Group D streptococci (the *Streptococcus bovis* group and enterococci) from other non-Group D streptococci. Ferric citrate is added as a source of oxidized iron to indicate a positive test.

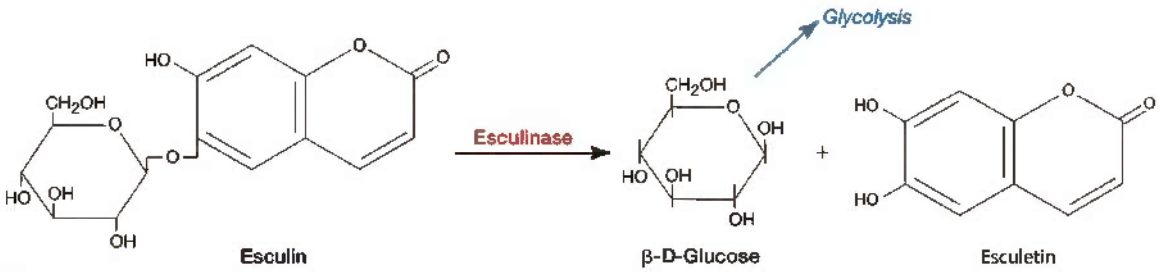
Esculin hydrolysis results in the production of D-glucose and esculetin (Fig. 4.3). The hydrolysis can occur under acidic conditions, or a β-glucosidase enzyme generically referred to as esculinase can catalyze it. Many bacteria possess esculinase, but the number of bacteria that are able to hydrolyze esculin in the presence of bile is much more limited, making this a useful selective and differential medium.

BEA can be prepared as a plated or a slanted medium. If the task is to isolate a streptococcus or enterococcus from a mixed culture, then a BEA plate is streaked for isolation and the differential results are read from that. If testing a pure culture for esculinase activity in the presence of bile, then a slanted medium is used.

When esculin is hydrolyzed in BEA, the resulting esculetin reacts with ferric ions in the medium to produce a dark brown/black precipitate (Fig. 4.4), which darkens the medium surrounding the growth. When a BEA plate is used (as in this lab exercise), an organism that darkens the medium even slightly is bile esculin-positive (Fig. 4.5). An organism that doesn't darken the medium is negative. A positive result is recorded on BEA slants when more than half of the medium is blackened. No blackening to less than half-blackened is considered a negative result.

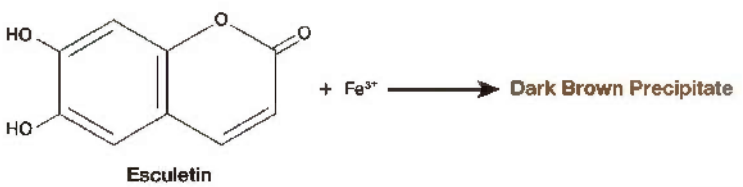


4.5 Bile Esculin Agar ♦ This plate was inoculated with two Gram-positive cocci. The one on the left is bile esculin positive and the one on the right is bile esculin negative.



4.3 Hydrolysis of Esculin ♦ Many organisms can hydrolyze esculin with the enzyme esculinase, but the Group D streptococci and enterococci are unique in their ability to do this in the presence of bile salts.

4.4 Bile Esculin Test Indicator Reaction ♦ Esculetin, produced during the hydrolysis of esculin, reacts with Fe^{3+} in the medium to produce a dark brown to black precipitate in the medium.



Application

BEA is used for the isolation and presumptive identification of bile esculin-positive enterococci (typically *Enterococcus faecalis* and *E. faecium*) and the bovis group of streptococci (Group D *Streptococcus bovis, S. equinus, S. gallolyticus,* and other streptococci) from non-Group D streptococci. It also can be used to distinguish the genera bile esculin-positive *Enterobacter, Klebsiella,* and *Serratia* from other genera in the *Enterobacteriaceae.*

In This Exercise

Today you will spot inoculate one bile esculin agar plate with two test organisms and incubate it for up to 48 hours. (In a clinical application, BEA plates would be streaked for isolation.) Any blackening of the medium before the end of 48 hours can be recorded as positive. Negative results must incubate the full 48 hours before final determination is made. Use Table 4-3 as a guide.

✓ Materials

Per Student Group

- ☐ One bile esculin agar plate
- ☐ Fresh broth cultures of:
  - *Lactococcus lactis* or *Streptococcus mutans*
  - *Enterococcus faecalis* (BSL-2) or *Streptococcus gallolyticus*

Medium Recipe

Bile Esculin Agar

• Pancreatic digest of gelatin	5.0 g
• Beef extract	3.0 g
• Oxgall (ox bile)	20.0 g
• Ferric citrate	0.5 g
• Esculin	1.0 g
• Agar	14.0 g
• Distilled or deionized water	1.0 L
pH 6.6–7.0 at 25°C	

Procedure

Lab One

1. Gently mix each culture according to your lab's standards.
2. Obtain a bile esculin agar plate.
3. Using a permanent marker, divide the bottom into two halves.
4. Label the plate with the organisms' names, your name, and the date.
5. Spot inoculate (refer to Appendix B) the sectors on the BEA plate with the test organisms. **Use BSL-2 precautions when transferring *E. faecalis*, if used.**
6. Invert and incubate the plate at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Examine the plate for any darkening of the medium.
2. Record your results on the data sheet, page 251.

References

Delost, Maria Dannessa. Page 132 in *Introduction to Diagnostic Microbiology.* St. Louis, MO: Mosby, 1997.

Lányi, B. Page 56 in *Methods in Microbiology,* Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

McFaddin, Jean F. Page 8 in *Biochemical Tests for Identification of Medical Bacteria,* 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Spellerberg, Barbara and Claudia Brandt. Page 342 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Tille, Patricia M. Page 199 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power. Page 156 in *Difco™ & BBL™ Manual–Manual of Microbiological Culture Media.* Sparks, MD: Becton, Dickinson and Co., 2003.

TABLE **4-3** **Bile Esculin Test Results and Interpretations**

Table of Results		
Result	**Interpretation**	**Symbol**
Medium is darkened within 48 hours	Presumptive identification as a member of Group D *Streptococcus* or *Enterococcus*	+
No darkening of the medium after 48 hours	Presumptive determination as not a member of Group D *Streptococcus* or *Enterococcus*	–

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 4-3

Bile Esculin Agar

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 4-3, page 250, when recording your results and interpretations in the table below.

Organism	Color Result	+/−	Interpretation and Presumptive ID

QUESTIONS

1 *In your own words, what is the application (purpose) of BEA as a plated medium?*

2 *Which ingredient(s) in BEA supply(ies)*

a. *Carbon?*

b. *Nitrogen?*

3 *Is BEA a defined or an undefined medium? Provide the reasoning behind your choice and explain why this formulation is desirable.*

4 *In your own words, what is the role of oxgall in BEA and how does it work?*

5 *In your own words, what is the role of esculin in BEA?*

6 *Would removal of oxgall (ox bile) from BEA alter the medium's sensitivity or specificity? (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

7 *Group D streptococci and the enterococci are found in the digestive tracts of various mammals. On a very general level, why might you expect them to give the BEA results they do?*

EXERCISE
4-4
Mannitol Salt Agar

Theory

Mannitol salt agar (MSA) contains the carbohydrate mannitol, 7.5% sodium chloride (NaCl), and the pH indicator phenol red. Phenol red is yellow below pH 6.8, red at pH 7.4 to 8.4, and pink at pH 8.4 and above. Mannitol provides the substrate for fermentation and makes the medium differential. Sodium chloride makes the medium selective because its concentration is high enough to dehydrate and kill most bacteria. Staphylococci thrive on the medium, largely because of their adaptation to salty habitats such as human skin. Phenol red indicates whether fermentation with an acid end-product has taken place by changing color as the pH changes. (See Section 5, A Word About Biochemical Tests and Acid-Base Reactions.)

Most staphylococci are able to grow on MSA, but do not ferment mannitol, so their growth appears pink or red and the medium remains unchanged. *Staphylococcus aureus* ferments mannitol, which produces acids and lowers the pH of the medium (Fig. 4.6). The result is formation of bright yellow colonies usually surrounded by a yellow halo (Fig. 4.7). For more information on fermentation, refer to Exercise 5-3 and Appendix A.

Application

Mannitol salt agar is used for isolation and differentiation of *Staphylococcus aureus* from other *Staphylococcus* species. It is not used to determine the ability of an isolate to ferment mannitol. A fermentation broth would be used instead (see Exercise 5-3).

In This Exercise

Today you will spot inoculate one MSA plate and one nutrient agar (NA) plate with three test organisms. (In a clinical application, MSA plates would be streaked for isolation.) The NA will serve as a comparison for growth quality on the MSA plate.

4

✓ Materials

Per Student Group

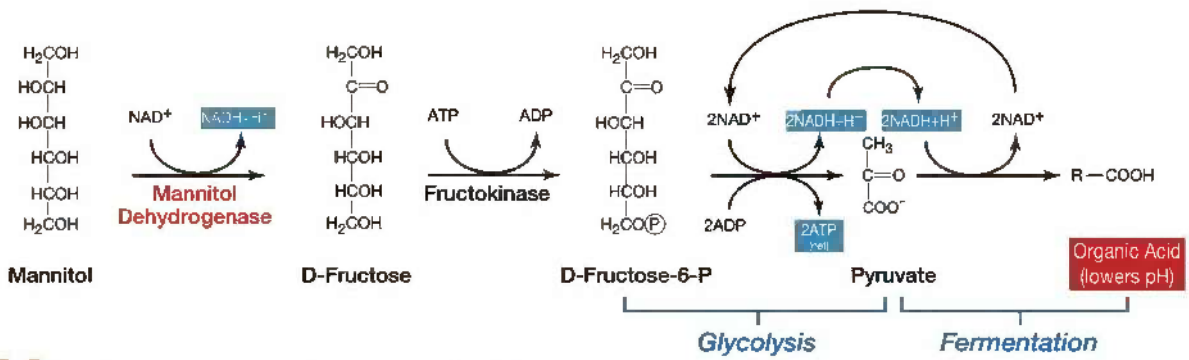
- ☐ One MSA plate
- ☐ One NA plate
- ☐ Fresh broth cultures of:
  - *Staphylococcus aureus* (BSL-2)
  - *Staphylococcus epidermidis*
  - *Escherichia coli*

Medium Recipes

Mannitol Salt Agar

• Beef extract	1.0 g
• Peptone	10.0 g
• Sodium chloride	75.0 g
• D-Mannitol	10.0 g
• Phenol red	0.025 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L

pH 7.2–7.6 at 25°C



4.6 Mannitol Fermentation with Acid End-Products ♦

4.7 Mannitol Salt Agar ♦ This medium is selective for members of the genus *Staphylococcus* and allows differentiation between those that ferment mannitol to acid end products (top) and those that do not (bottom).

Nutrient Agar

- Beef extract 3.0 g
- Peptone 5.0 g
- Agar 15.0 g
- Distilled or deionized water 1.0 L

pH 6.6–7.0 at 25°C

Procedure

Lab One

1. Gently mix each culture according to your lab's standards.
2. Using a permanent marker, divide the bottom of each plate into three sectors.
3. Label the plates with the organisms' names, your name, and the date.
4. Spot inoculate (refer to Appendix B) the sectors on the MSA plate with the test organisms. **Use BSL-2 precautions when transferring *S. aureus*.**
5. Repeat step 4 with the nutrient agar plate. Use the same positions for each specimen as used on the MSA plate.
6. Invert and incubate the plate at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Examine and compare the plates for color and quality of growth.
2. Record your results on the data sheet, page 255. Refer to Table 4-4 as needed.

References

Delost, Maria Dannessa. Page 112 in *Introduction to Diagnostic Microbiology, a Text and Workbook*. St. Louis, MO: Mosby, 1997.

Tille, Patricia M. Page 199 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power. Page 349 in *Difco™ & BBL™ Manual–Manual of Microbiological Culture Media*. Sparks, MD: Becton, Dickinson and Co., 2003.

TABLE **4-4** **Mannitol Salt Agar Results and Interpretations**

Table of Results		
Result	**Interpretation**	**Presumptive ID**
Poor growth (P) or no growth (N)	Organism is inhibited by NaCl	Not *Staphylococcus*
Good growth (G)	Organism is not inhibited by NaCl	*Staphylococcus*
Yellow growth or halo (Y)	Organism produces acid from mannitol fermentation	Possible pathogenic *Staphylococcus aureus*
Red growth (no halo) (R)	Organism does not ferment mannitol.	*Staphylococcus* other than *S. aureus*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 4-4

Mannitol Salt Agar

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 4-4, page 254, when recording your results and interpretations in the table below. Use abbreviations or symbols as needed.

Organism	Growth (N/P/G)		MSA Growth Color (Y/R)	Interpretation and Presumptive ID
	MSA	NA		

QUESTIONS

1 *In your own words, what is the application (purpose) of MSA?*

2 *Which ingredient(s) in MSA supply(ies)*

a. *Carbon?*

b. *Nitrogen?*

3 *Is MSA a defined or an undefined medium? Provide the reasoning behind your choice and explain why this formulation is desirable.*

4 *In your own words, what is the role of sodium chloride in MSA and how does it work?*

5 *In your own words, what are the roles of mannitol and phenol red in MSA?*

6 *Think about the results you recorded.*

a. *Growth on the MSA and NA plates was recorded as "good growth," "poor growth," or "no growth." These are qualitative and, at least for the first two, subjective terms. What did you use to establish what constituted "good growth?"*

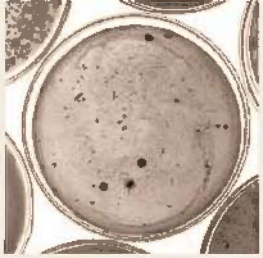
b. *Why wouldn't it be advisable to compare growth of the organisms on each plate to each other? There are at least two answers to this question!*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 4-4

(continued)

7 *Would removal of sodium chloride from MSA alter the medium's sensitivity or specificity? Explain your answer. (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

8 *Suppose a mistake is made in preparing a batch of MSA and the starting pH is 7.4 instead of 7.0–7.2. Would that affect the medium's sensitivity or specificity? Explain your answer. (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

9 *With the diversity of microorganisms in the world, how can a single test such as MSA be used to "confidently" identify* Staphylococcus aureus*? (***Hint:** *Consider the sample source and assumptions being made.)*

Selective Media for Isolation of Gram-negative Rods

Members of the family *Enterobacteriaceae*—the enteric "gut" bacteria—are commonly found in clinical samples. Depending on the circumstances, some organisms in a mixed sample are contaminants and relatively benign, whereas others are potentially harmful and must be isolated and identified. The media in this part of Section 4 are designed to isolate and differentiate these organisms from each other and to discourage growth of other organisms that are not currently of interest to the microbiologist.

The four examples selected for this unit are MacConkey agar, eosin methylene blue (EMB) agar, hektoen enteric (HE) agar, and xylose lysine desoxycholate (XLD) agar. MacConkey agar and EMB agar are selective for Gram-negative organisms and both contain indicators to differentiate lactose fermenters from lactose non-fermenters. EMB agar is commonly used to test for the presence of coliforms in environmental samples. As mentioned in the introduction to this section, the presence of coliforms in the environment suggests fecal contamination and the possible presence of enteric pathogens. HE agar differentiates *Salmonella* and *Shigella* from each other and from other enterics based on their ability to overcome the inhibitory effects of bile; reduce sulfur to H_2S; and ferment lactose, sucrose, and/or salicin. XLD agar favors growth of *Salmonella*, *Shigella*, or *Providencia* based on their ability to overcome the inhibitory effects of desoxycholate, and differentiates them based on their ability to reduce sulfur to H2S, decarboxylate the amino acid lysine, and their inability to ferment xylose, sucrose, or lactose. (For more information about fermentation or decarboxylation reactions, refer to Section 5, "A Word About Biochemical Tests and Acid-Base Reactions," and Exercises 5-3, 5-4, and 5-10. Also see Appendix A.) ◆

EXERCISE 4-5

MacConkey Agar

Theory

MacConkey agar is a commonly used selective and differential medium containing lactose, bile salts, neutral red, and crystal violet. Bile salts and crystal violet inhibit growth of Gram-positive bacteria. Neutral red dye is a pH indicator that is colorless above a pH of 6.8 and red at a pH less than 6.8. Organisms that ferment lactose to acid end-products lower the pH (Fig. 4.8) and their colonies turn a pink to red color. On occasion, a pink to red bile precipitate will also form in the agar due to the lowered pH. Lactose non-fermenters retain their normal color or the color of the medium (Fig. 4.9). Formulations without crystal violet allow growth of *Enterococcus* and some species of *Staphylococcus*, which ferment the lactose and appear pink on the medium.

Application

MacConkey agar is used to isolate and differentiate members of the *Enterobacteriaceae* based on the ability to ferment lactose. Variations on the standard medium include MacConkey agar w/o CV (without crystal violet) to allow growth of Gram-positive cocci, or MacConkey agar CS to control swarming bacteria (*Proteus*) that interfere with other results.

In This Exercise

You will spot inoculate one MacConkey agar plate and one nutrient agar (NA) plate with three test organisms. (In a clinical application, MacConkey plates would be streaked for isolation.) The NA plate will serve as a comparison for growth quality on the MacConkey agar plate.

✓ Materials

Per Student Group

- ☐ One MacConkey agar plate
- ☐ One nutrient agar plate
- ☐ Fresh broth cultures of:
  - *Escherichia coli*
  - *Providencia stuartii*
  - *Staphylococcus epidermidis*

Medium Recipes

MacConkey Agar

• Pancreatic digest of gelatin	17.0 g
• Pancreatic digest of casein	1.5 g
• Peptic digest of animal tissue	1.5 g
• Lactose	10.0 g
• Bile salts	1.5 g
• Sodium chloride	5.0 g
• Neutral red	0.03 g
• Crystal violet	0.001 g
• Agar	13.5 g
• Distilled or deionized water	1.0 L
pH 6.9–7.3 at 25°C	

Nutrient Agar

• Beef extract	3.0 g
• Peptone	5.0 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L
pH 6.6–7.0 at 25°C	

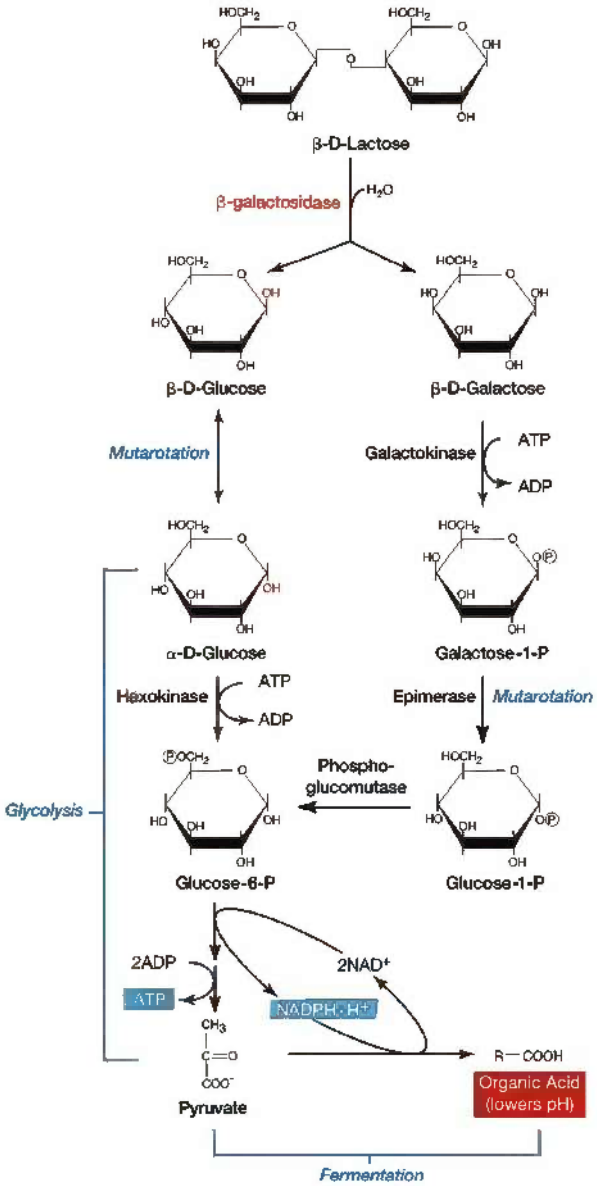
Procedure

Lab One

1. Gently mix each culture according to your lab's standards.
2. Using a permanent marker, divide the bottom of each plate into three sectors.
3. Label the plates with the organisms' names, your name, and the date.
4. Spot inoculate (refer to Appendix B) the sectors on the MacConkey agar plate with the test organisms.
5. Repeat step 4 with the nutrient agar plate. Use the same positions for each specimen as used on the MacConkey agar plate.
6. Invert and incubate the plate at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Examine and compare the plates for color and for quality of growth.
2. Refer to Table 4-5 when recording your results on the data sheet, page 263.



4.8 Lactose Fermentation with Acid End-Products ♦

4.9 MacConkey Agar ♦ This MacConkey Agar plate was inoculated with two Gram-positive cocci (left and right) and two members of the *Enterobacteriaceae* (Gram-negative rods—top and bottom). Obviously, the Gram positives were inhibited by the bile salts and crystal violet. The top Gram negative is a coliform and fermented lactose to acid end products and is pinkish. Also notice the bile precipitate around this growth. The lower Gram negative, a noncoliform, did not ferment lactose and appears its natural color.

TABLE 4-5 MacConkey Agar Results and Interpretations

Table of Results		
Result	**Interpretation**	**Presumptive ID**
Poor growth (P) or no growth (N)	Organism is inhibited by crystal violet and/or bile	Gram positive
Good growth (G)	Organism is not inhibited by crystal violet or bile	Gram negative
Pink to red growth with or without bile precipitate (R)	Organism produces acid from lactose fermentation	Probable coliform
Growth is "colorless" (not red or pink) (C)	Organism does not ferment lactose.	Noncoliform

References

Tille, Patricia M. Pages 86–87 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Washington C. et al. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Zimbro, Mary Jo and David A. Power. Page 334 in *Difco™ & BBL™ Manual–Manual of Microbiological Culture Media*. Sparks, MD: Becton, Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 4-5

MacConkey Agar

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 4-5, page 261, when recording your results and interpretations in the table below. Use abbreviations or symbols as needed.

Organism	Growth (N/P/G)		MSA Growth Color (R/C)	Interpretation and Presumptive ID
	MAC	NA		

QUESTIONS

1 *In your own words, what is the application (purpose) of MacConkey agar?*

2 *Which ingredient(s) in MacConkey agar supply(ies)*

a. *Carbon?*

b. *Nitrogen?*

3 *Is MacConkey agar a defined or an undefined medium? Provide the reasoning behind your choice and explain why this formulation is desirable.*

4 *In your own words, what are the roles of crystal violet and bile salts in MacConkey agar?*

5 *In your own words, what are the roles of neutral red and lactose in MacConkey agar?*

6 *Think about the results you recorded.*

a. *Growth on the MacConkey agar and NA plates was recorded as "good growth," "poor growth," or "no growth." These are qualitative and, at least for the first two, subjective terms. What did you use to establish what constituted "good growth?"*

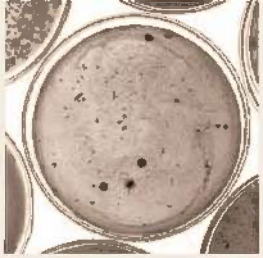
b. *Why wouldn't it be advisable to compare growth of the organisms on each plate to each other? There are at least two answers to this question!*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 4-5

(continued)

7 *Would removing bile salts and/or crystal violet from MacConkey agar alter the medium's sensitivity or specificity? Explain your answer. (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

8 *Suppose a mistake is made in preparing a batch of MacConkey agar and the starting pH is 7.6 instead of 6.9–7.3. Would that affect the medium's sensitivity or specificity? Explain your answer. (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

9 *Compare the recipes of nutrient agar and MacConkey agar. If an organism can grow on both media, on which would you expect it to grow better? Explain your answer.*

EXERCISE
4-6

Eosin Methylene Blue Agar (Levine)

Theory

Eosin methylene blue (EMB) agar is a complex (chemically undefined), selective, and differential medium. It contains digest of gelatin, lactose, and the dyes eosin Y and methylene blue. The gelatin provides organic carbon, and nitrogen. Lactose is fermented to acid end-products by coliforms such as *Escherichia coli* and *Enterobacter aerogenes*, whereas it is not by pathogens such as *Proteus*, *Shigella*, and *Salmonella* species.

The purpose of the dyes is twofold: (1) they inhibit the growth of most Gram-positive organisms (some *Enterococcus* and *Staphylococcus* species are exceptions), and (2) they react with vigorous lactose fermenters whose acid end-products turn the growth dark purple or black. This dark growth is typical of *Escherichia coli* and is usually accompanied by a green metallic sheen (Fig. 4.10). Other, less-aggressive lactose fermenters, such as *Enterobacter* or *Klebsiella* species, produce colonies that can range from pink to dark purple on the medium. Lactose non-fermenters typically retain their normal color or take on the coloration of the medium.

Application

EMB agar (Levine) is used for the isolation of fecal coliforms. It can be streaked for isolation or used in the Membrane Filter Technique as discussed in Exercise 8-12.

In This Exercise

You will spot inoculate one EMB agar plate and one nutrient agar (NA) plate with four test organisms. The NA plate will serve as a comparison for growth quality on the EMB agar plate.

✓ Materials

Per Student Group

- ☐ One EMB plate
- ☐ One NA plate
- ☐ Fresh broth cultures of:
  - *Enterobacter aerogenes*
  - *Enterococcus faecalis* (BSL-2)
  - *Escherichia coli*
  - *Providencia stuartii*

4.10 Eosin Methylene Blue Agar ♦ This EMB plate was inoculated with two Gram-positive cocci (left and right) and two members of the *Enterobacteriaceae*, a coliform at the top and a noncoliform at the bottom. Fermentation of lactose by the coliform produced a shiny green appearance to the growth. The noncoliform was not inhibited by the dyes but also did not ferment the lactose. The Gram-positive coccus on the left barely grew and growth of the one on the right was completely inhibited.

Medium Recipes

Eosin Methylene Blue Agar (Levine)

• Pancreatic digest of gelatin	10.0 g
• Lactose	10.0 g
• Dipotassium phosphate	2.0 g
• Methylene blue	0.065 g
• Eosin Y	0.4 g
• Agar	13.5 g
• Distilled or deionized water	1.0 L
pH 6.9–7.3 at 25°C	

Nutrient Agar

• Beef extract	3.0 g
• Peptone	5.0 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L
pH 6.6–7.0 at 25°C	

Procedure

Lab One

1. Gently mix each culture according to your lab's standards.
2. Using a permanent marker, divide the bottom of each plate into four sectors.
3. Label the plates with the organisms' names, your name, and the date.
4. Spot inoculate (refer to Appendix B) the sectors on the EMB plate with the test organisms. **Use BSL-2 precautions when transferring *E. faecalis*.**
5. Repeat step 4 with the nutrient agar plate. Use the same positions for each specimen as used on the EMB plate.
6. Invert and incubate the plate at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Examine and compare the plates for color and quality of growth.
2. Record your results on the data sheet, page 269. Refer to Table 4-6 as needed.

References

Tille, Patricia M. Page 84 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Washington C. et al. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Zimbro, Mary Jo and David A. Power. Page 218 in *Difco™ & BBL™ Manual–Manual of Microbiological Culture Media*. Sparks, MD: Becton, Dickinson and Co., 2003.

TABLE 4-6 **EMB Results and Interpretations**

Table of Results		
Result	**Interpretation**	**Presumptive ID**
Poor growth (P) or no growth (N)	Organism is inhibited by eosin and methylene blue	Gram positive
Good growth (G)	Organism is not inhibited by eosin and methylene blue	Gram negative
Growth is pink and mucoid (Pi)	Organism ferments lactose with little acid production	Possible coliform
Growth is "dark" (purple to black, with or without green metallic sheen) (D)	Organism ferments lactose and/or sucrose with acid production	Probable coliform
Growth is "colorless" (not red or pink) (C)	Organism does not ferment lactose or sucrose.	Noncoliform

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 4-6

Eosin Methylene Blue Agar (Levine)

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 4-6, page 268, when recording your results and interpretations in the table below. Use abbreviations or symbols as needed.

Organism	Growth (N/P/G)		EMB Growth Color (Pi/D/C)	Interpretation and Presumptive ID
	EMB	NA		

QUESTIONS

1 *In your own words, what is the application (purpose) of EMB agar?*

2 *Which ingredient(s) in EMB agar supply(ies)*

a. *Carbon?*

b. *Nitrogen?*

3 *Is EMB agar a defined or an undefined medium? Provide the reasoning behind your choice and explain why this formulation is desirable.*

4 *In your own words, what are the roles of eosin Y and methylene blue in EMB agar?*

5 *In your own words, what is the role of lactose in EMB agar?*

6 *Think about the results you recorded.*

a. *Growth on the EMB agar and NA plates was recorded as "good growth," "poor growth," or "no growth." These are qualitative and, at least for the first two, subjective terms. What did you use to establish what constituted "good growth?"*

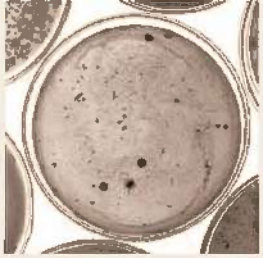
b. *Why wouldn't it be advisable to compare growth of the organisms on each plate to each other? There are at least two answers to this question!*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 4-6

(continued)

7 *Would removing eosin Y and/or methylene blue from EMB agar alter the medium's sensitivity or specificity? Explain your answer. (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

8 *Suppose a mistake is made in preparing a batch of EMB agar and the starting pH is 7.6 instead of 6.9–7.3. Would that affect the medium's sensitivity or specificity? Explain your answer. (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

9 *Compare the recipes of nutrient agar and EMB agar. If an organism can grow on both media, on which would you expect it to grow better? Explain your answer.*

EXERCISE
4-7

Hektoen Enteric Agar

Theory

Hektoen enteric (HE) agar is a complex (chemically undefined), moderately selective, and differential medium designed to isolate *Salmonella* and *Shigella* species from other enterics. The test is based on the ability to ferment lactose, sucrose, or salicin to acid end-products, and to reduce sulfur (e.g., sodium thiosulfate) to hydrogen sulfide gas (H_2S). Ferric ammonium citrate is included as a source of oxidized iron to react with any H_2S produced to form the black precipitate ferrous sulfide (FeS). Bile salts are included to prevent or inhibit growth of Gram-positive organisms. The bile salts also have a moderate inhibitory effect on enterics, so relatively high concentrations of animal tissue and yeast extract are included to offset this situation. Bromothymol blue and acid fuchsin dyes are added to indicate pH changes.

Differentiation is possible as a result of the various colors produced in the colonies and in the agar. Enterics that produce acid from fermentation will produce yellow to salmon-pink colonies. Neither *Salmonella* nor *Shigella* species ferment any of the sugars; instead they break down the animal tissue, which raises the pH of the medium slightly and gives their colonies a blue-green color. Additionally, *Salmonella* species reduce sulfur to H_2S, so the colonies formed also contain FeS, which makes them partially or completely black. Refer to Figure 4.11.

Application

HE agar is used to isolate and differentiate *Salmonella* and *Shigella* species from other Gram-negative enteric organisms.

In This Exercise

You will streak inoculate one Hektoen enteric agar plate and one nutrient agar plate with four test organisms. (In a clinical application, the agar would be streaked for isolation with a patient's sample.)

The nutrient agar will serve as a comparison for growth quality on the Hektoen enteric agar plate.

4.11 Hektoen Enteric Agar ◆ HE agar is selective for Gram negatives and is typically used to isolate members of the *Enterobacteriaceae*. It differentiates between coliforms, which produce acid from lactose fermentation, and noncoliforms, and identifies sulfur reducers. This Hektoen enteric agar was inoculated with four Gram-negative rods. At the top is a coliform, which produced acid from lactose fermentation resulting in a yellow (or sometimes salmon) color. To the right is a noncoliform that also is negative for sulfur reduction, as evidenced by the blue green color. The other two organisms are noncoliforms that are positive for sulfur reduction, resulting in their black color.

✓ Materials

Per Student Group

- ☐ One HE agar plate
- ☐ One NA plate
- ☐ Fresh broth cultures of:
  - *Enterococcus faecalis* (BSL-2)
  - *Escherichia coli*
  - *Salmonella typhimurium* (BSL-2)
  - *Shigella flexneri* (BSL-2) or *Providencia stuartii*

Medium Recipes

Hektoen Enteric Agar

• Yeast extract	3.0 g
• Peptic digest of animal tissue	12.0 g
• Lactose	12.0 g
• Sucrose	12.0 g
• Salicin	2.0 g
• Bile salts	9.0 g
• Sodium chloride	5.0 g
• Sodium thiosulfate	5.0 g
• Ferric ammonium citrate	1.5 g
• Bromothymol blue	0.064 g
• Acid fuchsin	0.1 g
• Agar	13.5 g
• Distilled or deionized water	1.0 L
pH 7.4–7.8 at 25°C	

Nutrient Agar

• Beef extract	3.0 g
• Peptone	5.0 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L
pH 6.6–7.0 at 25°C	

Procedure

Lab One

1. Gently mix each culture according to your lab's standards.
2. Using a permanent marker, divide the bottom of each plate into four sectors.
3. Label the plates with the organisms' names, your name, and the date.
4. Spot inoculate (refer to Appendix B) the sectors on the HE plate with the test organisms. **Use BSL-2 precautions when transferring *E. faecalis*, *S. typhimurium*, and *S. flexneri* (if used).**
5. Repeat step 4 with the nutrient agar plate. Use the same positions for each specimen as used on the HE plate.
6. Invert and incubate the plate at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Examine and compare the plates for color and quality of growth.
2. Record your results on the data sheet, page 275. Refer to Table 4-7 as needed.

References

Tille, Patricia M. Page 86 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Washington C. et al. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Zimbro, Mary Jo and David A. Power. Page 265 in *Difco™ & BBL™ Manual–Manual of Microbiological Culture Media*. Sparks, MD: Becton, Dickinson and Co., 2003.

TABLE **4-7** **Hektoen Enteric Agar Results and Interpretations**

Table of Results		
Result	**Interpretation**	**Presumptive ID**
Poor growth (P) or no growth (N)	Organism is inhibited by bile and/or one of the dyes included	Gram positive
Good growth (G)	Organism is not inhibited by bile or any of the dyes included	Gram negative
Pink to orange growth (Pi)	Organism produces acid from lactose fermentation	Not *Shigella* or *Salmonella*
Blue-green growth with black precipitate (Bppt)	Organism does not ferment lactose, but reduces sulfur to hydrogen sulfide (H_2S)	Probable *Salmonella*
Blue-green growth without black precipitate (B)	Organism does not ferment lactose or reduce sulfur.	Probable *Shigella* or rarely *Salmonella*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 4-7

Hektoen Enteric Agar

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 4-7, page 274, when recording your results and interpretations in the table below. Use abbreviations or symbols as needed.

Organism	Growth (N/P/G)		EMB Growth Color (Pi/Bppt/B)	Interpretation and Presumptive ID
	HE	NA		

QUESTIONS

1 *In your own words, what is the application (purpose) of HE agar?*

2 *Which ingredient(s) in HE agar supply(ies)*

a. *Carbon?*

b. *Nitrogen?*

3 *Is HE agar a defined or an undefined medium? Provide the reasoning behind your choice and explain why this formulation is desirable.*

4 *In your own words, what is the role of bile salts in HE agar?*

5 *In your own words, what are the roles of lactose, sucrose, and salicin in HE agar?*

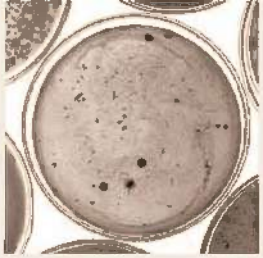
6 *In your own words, what are the roles of acid fuchsin and bromothymol blue in HE agar?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 4-7

(continued)

7 *Think about the results you recorded.*

a. *Growth on the HE agar and NA plates was recorded as "good growth," "poor growth," or "no growth." These are qualitative and, at least for the first two, subjective terms. What did you use to establish what constituted "good growth?"*

b. *Why wouldn't it be advisable to compare growth of the organisms on each plate to each other? There are at least two answers to this question!*

8 *Would removing bile salts from HE agar alter the medium's sensitivity or specificity? Explain your answer. (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

9 *Suppose a mistake is made in preparing a batch of HE agar and the starting pH is 7.0 instead of 7.4–7.8. Would that affect the medium's sensitivity or specificity? Explain your answer. (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

4 10 *Compare the recipes of nutrient agar and HE agar. If an organism can grow on both media, on which would you expect it to grow better? Explain your answer.*

11 *All enterics ferment glucose. What would be some consequences of replacing the sugars in HE agar with glucose? What color combinations would you expect to see?*

12 *Write a description of an organism whose colonies are green with black centers on HE agar.*

Xylose Lysine Desoxycholate Agar

EXERCISE 4-8

Theory

Xylose lysine desoxycholate (XLD) agar is a selective and differential medium containing sodium desoxycholate, xylose, lactose, sucrose, lysine, sodium thiosulfate, phenol red, and ferric ammonium citrate. Desoxycholate is a bile salt that inhibits growth of Gram-positive organisms. Xylose, lactose, and sucrose are sugars provided for fermentation; and lysine, an amino acid, is provided for decarboxylation (removal of the carboxyl group–COOH, see Exercise 5-10). Sodium thiosulfate provides oxidized sulfur for organisms capable of reducing it to hydrogen sulfide gas (H_2S). Ferric ammonium citrate performs as an indicator because it releases ferric ions (Fe^{3+}) into the medium that will react with H_2S and form ferrous sulfide (FeS), a black precipitate. Phenol red, which is yellow when acidic and red or pink when alkaline, is added as a pH indicator.

Organisms that ferment xylose will acidify the medium and produce yellow colonies (see Section 5, "A Word About Biochemical Tests and Acid-Base Reactions"). Organisms able to decarboxylate lysine will release CO_2 into the medium, raise the pH, and produce red colonies (see Exercise 5-10). Organisms able to reduce sulfur will produce H_2S, which will precipitate with Fe^{3+} and form black colonies.

Shigella and *Providencia* species, which do not ferment xylose, lactose, or sucrose but decarboxylate lysine, form red colonies on the medium. *Salmonella* species reduce sulfur, ferment xylose (but not lactose or sucrose), and decarboxylate lysine. The fermentation acidifies the medium, but because the xylose is in relatively short supply the lysine decarboxylation raises the pH and produces red colonies with black centers. Other enterics (*Escherichia, Klebsiella, Enterobacter*) that would ordinarily deplete the small amount of xylose in the medium and then raise the pH by decarboxylating the lysine, are discouraged from doing so by the ample supply of the other fermentable sugars, lactose and sucrose. These organisms typically produce yellow colonies on the medium (Fig. 4.12).

Application

XLD agar is a selective and differential medium used to isolate and identify *Shigella* and *Providencia* from stool samples.

4.12 Xylose Lysine Desoxycholate Agar ♦ XLD agar is a selective medium frequently used to isolate species of *Enterobacteriaceae*. It is also a differential medium that detects acid from xylose fermentation, lysine decarboxylation, and sulfur reduction. At the top is a xylose fermenter, which has turned the medium (and itself) yellow. At the bottom is an organism negative for xylose fermentation and sulfur reduction, but positive for lysine decarboxylation, as evidenced by the red color. To the left and right are two xylose nonfermenters that decarboxylate lysine (note the red around the edges). Their black color means they are positive for sulfur reduction.

In This Exercise

You will spot inoculate one XLD agar plate and one nutrient agar (NA) plate with four test organisms. (In a clinical application, XLD would be streaked for isolation with a patient's sample.) The NA plate will serve as a comparison for growth quality on the XLD agar plate.

✓ Materials

Per Student Group

- ☐ One XLD agar plate
- ☐ One NA plate
- ☐ Fresh broth cultures of:
  - *Escherichia coli*
  - *Providencia stuartii*
  - *Salmonella typhimurium* (BSL-2)
  - *Staphylococcus epidermidis*

Medium Recipe

Xylose Lysine Desoxycholate Agar

• Xylose	3.5 g
• L-Lysine	5.0 g
• Lactose	7.5 g
• Sucrose	7.5 g
• Sodium chloride	5.0 g
• Yeast extract	3.0 g
• Phenol red	0.08 g
• Sodium desoxycholate	2.5 g
• Sodium thiosulfate	6.8 g
• Ferric ammonium citrate	0.8 g
• Agar	13.5 g
• Distilled or deionized water	1.0 L
pH 7.3–7.7 at 25°C	

Nutrient Agar

• Beef extract	3.0 g
• Peptone	5.0 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L
pH 6.6–7.0 at 25°C	

Procedure

Lab One

1. Gently mix each culture according to your lab's standards.
2. Using a permanent marker, divide the bottom of each plate into four sectors.
3. Label the plates with the organisms' names, your name, and the date.
4. Spot inoculate (refer to Appendix B) the sectors on the XLD plate with the test organisms. **Use BSL-2 precautions when transferring *S. typhimurium*.**
5. Repeat step 4 with the nutrient agar plate. Use the same positions for each organism as used on the XLD plate.
6. Invert and incubate the plate at 35 ± 2°C for 24 to 48 hours. (Be sure to remove the plates from the incubator at 24 hours. Reversions from fermentation to decarboxylation, due to exhaustion of sugar in the medium with the resulting yellow-to-red color shift, may lead to false identification as *Shigella* or *Providencia*.)

Lab Two

1. Examine and compare the plates for color and quality of growth.
2. Record your results on the data sheet, page 281. Refer to Table 4-8 as needed.

References

Forbes, Betty A., Daniel F. Sahm, and Alice S. Weissfeld. Chap. 10 in *Bailey & Scott's Diagnostic Microbiology*, 11th ed. St. Louis, MO: Mosby, 2002.

Tille, Patricia M. Page 88 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power. Page 625 in *Difco™ & BBL™ Manual–Manual of Microbiological Culture Media*. Sparks, MD: Becton, Dickinson and Co., 2003.

TABLE **4-8** **XLD Results and Interpretations**

Table of Results		
Result	**Interpretation**	**Presumptive ID**
Poor growth (P)	Organism is inhibited by desoxycholate	Gram positive
Good growth (G)	Organism is not inhibited by desoxycholate	Gram negative
Growth is yellow (Y)	Organism produces acid from xylose (and/or lactose, and/or sucrose) fermentation (A)	Not *Shigella* or *Providencia*
Red growth with black center (RB)	Organism reduces sulfur to hydrogen sulfide (H_2S)	Not *Shigella* or *Providencia* (probable *Salmonella*)
Red growth without black center (R)	Organism does not ferment xylose or ferments xylose slowly; alkaline products from lysine decarboxylation (K)	Probable *Shigella* or *Providencia*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 4-8

Xylose Lysine Desoxycholate Agar

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 4-8, page 280, when recording your results and interpretations in the table below. Use abbreviations or symbols as needed.

Organism	Growth (P/G)		EMB Growth Color (Y/RB/R)	Interpretation and Presumptive ID
	XLD	NA		

QUESTIONS

1 *In your own words, what is the application (purpose) of XLD agar?*

2 *Which ingredient(s) in XLD agar supply(ies)*

a. *Carbon?*

b. *Nitrogen?*

3 *Is XLD agar a defined or an undefined medium? Provide the reasoning behind your choice and explain why this formulation is desirable.*

4 *In your own words, what is the role of sodium desoxycholate in XLD agar?*

5 *In your own words, what are the roles of xylose, lactose, and sucrose in XLD agar?*

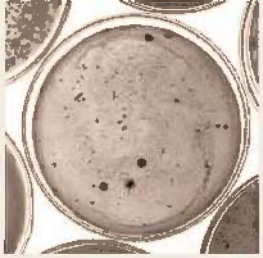
6 *In your own words, what is the role of phenol red in XLD agar?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 4-8

(continued)

7 *Think about the results you recorded.*

a. *Growth on the XLD agar and NA plates was recorded as "poor growth" or "good growth." These are qualitative and subjective terms. What did you use to establish what constituted "good growth?"*

b. *Why wouldn't it be advisable to compare growth of the organisms on each plate to each other? There are at least two answers to this question!*

8 *Would removing sodium desoxycholate from XLD agar alter the medium's sensitivity or specificity? Explain your answer. (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

9 *Suppose a mistake is made in preparing a batch of XLD Agar and the starting pH is 6.8 instead of 7.3–7.7. Would that affect the medium's sensitivity or specificity? Explain your answer. (See pages 8 and 9, "A Word About Experimental Design," for assistance with these terms.)*

10 *Compare the recipes of nutrient agar and XLD agar. If an organism can grow on both media, on which would you expect it to grow better? Explain your answer.*

11 *All enterics ferment glucose. What would be some consequences of replacing the sugars in XLD agar with glucose? What color combinations would you expect to see?*

12 *Write a description of an organism whose colonies are yellow with black on XLD agar.*

13 Shigella *and* Providencia *species virtually never ferment xylose, lactose, or sucrose. Because XLD is designed to directly identify these organisms, why were three unusable carbohydrates included when only one would seem to accomplish the goal?*

SECTION 5

Differential Tests

In the last century, bacteria have been differentiated based on their enormous biochemical diversity. The diagnostic systems that microbiologists use for this purpose are called **differential tests** or **biochemical tests** because they differentiate and sometimes identify microorganisms based on specific biochemical characteristics. As you will see repeatedly in this section, microbial biochemical characteristics are brought about by enzymatic reactions that are part of specific and well-understood metabolic pathways. The tests used in this section were selected to illustrate those pathways and the means to identify them.

The first half of Section Five will introduce you to the tests individually so you can familiarize yourself with the metabolic pathways and the mechanisms by which specific tests expose them. The second half offers the opportunity for you to use some multiple test media designed for rapid identification.

The categories of differential tests in this section are:

- Energy metabolism, including fermentation of carbohydrates and aerobic and anaerobic respiration
- Utilization of a specified medium component
- Decarboxylation and deamination of amino acids
- Hydrolytic reactions requiring intracellular or extracellular enzymes
- Multiple reactions performed in a single combination medium
- Miscellaneous differential tests

The section ends with an exercise in which you will isolate two unknown bacteria from a mixed culture, grow them in pure culture, and identify them. Thus, you will be employing skills developed in Section 1 (streak plate), Section 3 (Gram stain and microscope use), and apply appropriate differential tests from this section in the process. You will be doing what many microbiologists do on a daily basis!

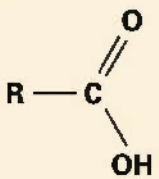
A Word about Biochemical Tests and Acid-Base Reactions

Differential test media provide limited specific growth conditions (usually including a single key substrate such as glucose or lactose in the case of fermentation media) whereby an organism can carry out a specified metabolic reaction or series of reactions of interest to the microbiologist. But however complex the chemistry may be, the evaluation of the test is often a simple check to see if an acid or base has been formed. Considering the diversity of physiological characteristics bacteria display, it is comforting to know that many of the biochemical tests used to differentiate them rely on a simple qualitative evaluation of pH! (For more about pH, see Exercise 2-10.) An indicator dye (e.g., phenol red, bromcresol purple, or bromothymol blue) is usually included in the test medium (or may be added after incubation) that changes color when the pH changes. The reason for the different dyes is that each one operates best in a specific pH range and allows media to be tailored to the type of organism being tested. Many oral bacteria, for example, prefer a slightly acidic environment (around pH 6) whereas enteric bacteria prefer a more neutral environment (around pH 7). Obviously, there are more metabolic reactions than can be discussed in this manual, but it is helpful to know a little bit about why certain metabolic products are formed and how they affect their environment.

Change in pH is an excellent indicator of what products (or kinds of products) are formed during metabolic activity. As such, it is an extremely useful tool in diagnostic microbiology. The principle is simple—if the pH changes (as evidenced by a color change) in one direction, the organism is said to have done one thing; if the pH (color) changes in the other direction, the organism is said to have done something else. If there is no change in color, it is determined that there was no reaction or *that the organism did not grow*. Naturally, the conditions of each biochemical test affect how the pH change is interpreted.

Biochemical media allow microbiologists to observe a variety of microbial metabolic pathways, but as you will see throughout this section, the one recurring theme is fermentation. Microbial fermentations produce three principal products: gas, alcohol, and/or acid. Gas (frequently in the form of CO_2) can be an easy indicator of fermentation because it is easy to see. Typically, this is accomplished by the addition of a small inverted test tube, called a Durham tube, to broth media. When gas-producing fermentation occurs, microscopic gas bubbles are trapped inside the Durham tube, which coalesce to form a visible gas bubble. Figure A.5 illustrates several fermentation pathways that use reactions where carbon compounds are split and CO_2 is released. Alcohols produced by fermentation are liquid and colorless, so they are not seen with the naked eye, and because they do not ionize in water they have minimal effect (if any) on the medium's pH. The weak acids (e.g., acetic acid, propionic acid, formic acid, succinic acid, and butyric acid) have a dramatic effect on the pH of any medium and can easily be detected by addition of one of the pH indicators mentioned above.

Acids, as defined by the Brønsted/Lowry theory of acids and bases, are substances that donate hydrogen ions (H^+) while bases are substances that accept hydrogen ions. All of the above-mentioned acids are called **carboxylic acids** because they contain the carboxyl functional group:



where "R" is the remainder of the molecule. (This may also be represented as R–COOH because it fits on a line of text better!) Because carboxylic acids can exist as either R–COOH or $R–COO^-$ in the same solution, they form a **conjugate pair**, where R–COOH is the conjugate acid and $R–COO^-$ is the conjugate base. A typical reaction would look like this: $R–COOH + H_2O \longrightarrow R–COO^- + H_2O + H^+$. As you can see, when the reaction goes to the right, hydrogen ions are released into the medium and this lowers the pH (Exercise 2-10).

Conversely, the removal of hydrogen ions or the addition of hydroxide ions (OH^-) to the water raises the pH. A typical way that an organism raises the pH in fermentation and other growth media is by breaking down proteins. Breaking down proteins raises the pH of media because the reaction releases ammonia (**deamination**), which further reacts with the water to form ammonium and hydroxide ions. The overall reaction looks like this:
$NH_3 + H_2O \longrightarrow NH_4^+ + OH^-$.

It is important to note that a differential medium prepared in the laboratory is not intended to duplicate an organism's natural environment. Differential media are artificial mixtures of chemicals and organic substrates intended to exploit different microbial abilities to perform in ways that might never actually be expressed in nature. So even though an organism may never radically lower the pH of its natural environment (because of low substrate availability or competing protein catabolism) we can manipulate it to do so in the lab by cultivating it in medium spiked with a specific sugar or other substrate. And when two or more species, otherwise indistinguishable by way of staining, microscopy, or colony characteristics, are thus compared, their differences frequently become clear. ◆

Introduction to Energy Metabolism Tests

Heterotrophic bacteria obtain their energy by performing either **respiration** or **fermentation** using ingested organic molecules. Both catabolic systems oxidize the organic molecules and transfer their chemical energy into high-energy bonds in **adenosine triphosphate (ATP)**. They differ principally in what **final electron acceptor (FEA)** is used, the presence or absence of an **electron transport chain (ETC)**, and in their ATP yield. Fermentations are characterized by the absence of an ETC and using an organic compound as the FEA, whereas respirations use an inorganic compound and possess an ETC. Oxygen serves as the FEA in **aerobic respiration**, whereas another inorganic compound (e.g., NO_3 or SO_4) acts in that role in **anaerobic respiration**.

Respiration accomplishes ATP synthesis by means of three well-understood pathways: **glycolysis**, the **citric acid cycle** (also known as **Krebs**, or **tricarboxylic acid cycle**), and **oxidative phosphorylation**, performed by an ETC. Fermentation pathways are many and varied, but all use the process of **substrate-level phosphorylation** to make ATP, in which the energy for adding the phosphate comes directly from breaking a bond in an organic molecule.

In both pathways, certain reactions require coenzymes during the oxidation of carbon intermediates. Coenzymes are the part of the enzyme-coenzyme-substrate complex that directly takes part in the transfer of electrons with the substrate. Because they can exist in either the oxidized or reduced state, coenzymes are responsible for both oxidations and reductions. The relevant coenzymes are nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD)

Glycolysis (Fig. A.1) is a sequence of reactions that results in splitting the six-carbon glucose molecule into two three-carbon **pyruvate** molecules while reducing NAD^+ to NADH and producing ATP by substrate phosphorylation. Both respiration and fermentation begin with glycolysis in most organisms. With respect to the carbon reactions, it is the fate of pyruvate that separates the two.

Following glycolysis is a complex reaction (the Entry Step–Fig. A.4) in which pyruvate is oxidized, one CO_2 molecule is removed and the remaining two-carbon fragment is attached to coenzyme A to produce **acetyl-CoA;** and NAD becomes reduced. The acetyl-CoA then enters the citric acid cycle (Fig. A.4) and is converted to two CO_2 molecules in a circular pathway of carbon reactions. These reactions release energy used to produce more ATP and in the process reduce many coenzymes. If both pyruvates follow this path, then all six carbons of glucose have become oxidized to CO_2.

If respiration were to end at this point, the cell would lose its ability to oxidize glucose because of a relative shortage of oxidized coenzymes. However, as mentioned above, cells perform another critical set of reactions called the electron transport chain. The ETC is a system whereby reduced coenzymes donate electrons to molecules that pass them down a chain of other molecules in a series of oxidation-reduction reactions to an inorganic FEA. As stated above, when the FEA is oxygen, the respiration is aerobic. When the final electron acceptor is an inorganic molecule other than oxygen (e.g., sulfate or nitrate) the respiration is anaerobic. Most ATP produced during respiration comes from the ETC.

Fermenting cells needs oxidized NAD to keep glycolysis operating, because that is its only method of ATP production, but they do not have an ETC. As an alternative, they use pyruvate or one of its derivatives as their FEA, resulting in reduced carbon compounds in the form of acids and organic solvents, as well as CO_2—the typical end products of fermentation. ◆

Reduction Potential

EXERCISE 5-1

Theory

All physical and chemical processes obey the Second Law of Thermodynamics—the law of **entropy**. Without constant energy input, order evolves to disorder, and high energy dissipates and becomes low energy. There is evidence of this everywhere—batteries run down, paint fades and peels, automobiles rust, fences fall down, and so on. In living systems, however, when there is an ample supply of energy, losses and gains of energy are often cyclic (though not equal)—energy lost in one reaction is captured in another.

The energy contained in a cellular molecule, called its **energy of formation**, comes from the energy released during reactions involved in its assembly from individual components. And again, obeying the law of entropy, *some* of this energy is retained by the molecule and can be released in subsequent reactions. The energy released in metabolic reactions is called **free energy**. Among other things, free energy is used to produce high-energy molecules such as ATP. (*Note*: Although ATP is a high-energy molecule, its energy content is much lower than that of the molecules in the metabolic pathways that produce it. Interestingly, compared to the pathway molecules, ATP has more readily *usable* energy. This means that a lot of unusable energy must be spent to build a relatively small amount of usable energy. So although you may not always hear words such as entropy to describe metabolism, this unavoidable loss of energy is precisely what drives the process.)

Recall from previous chemistry and/or biology classes, oxidation-reduction, or "redox" reaction, plays a major role in metabolic processes. Remember that oxidation is defined as the loss of electrons and reduction is the gain of electrons. A molecule that gives up electrons becomes oxidized, but is called a reductant because it is reducing another molecule. Similarly, a molecule that accepts electrons is an oxidant. Free energy released during redox reactions is the focus of this exercise.

Free energy released from redox reactions is heat energy that can be both measured and calculated.[1] Using a visual aid, such as the electron tower in Figure 5.1, allows visualization and predictions involving electron exchanges between molecules. Electron towers visually represent the relationship of many important cellular oxidants and reductants in terms of their various reduction potentials. **Reduction potential**, simply defined, is the tendency (measured in volts) of molecules to donate electrons.

Redox Pair	Reduction Potential (E_o')	
CO_2/glucose	−0.43V	(24e⁻)
$2H^+/H_2$	−0.42V	(2e⁻)
$S_2O_3^{2-}/HS^-$	−0.40V	(2e⁻)
NAD^+/NADH	−0.32V	(2e⁻)
S^0/H_2S	−0.28V	(2e⁻)
SO_4^{2-}/H_2S	−0.22V	(8e⁻)
Pyruvate/lactate	−0.19V	(2e⁻)
Fumarate/succinate	+0.03V	(2e⁻)
Cytochrome $b_{ox/red}$	+0.035V	(1e⁻)
Fe^{3+}/Fe^{2+} (pH 7)	+0.20V	(1e⁻)
$Ubiquinone_{ox/red}$	+0.11V	(2e⁻)
Cytochrome $c_{ox/red}$	+0.25V	(1e⁻)
Cytochrome $a_{ox/red}$	+0.39V	(1e⁻)
NO_3^-/NO_2^-	+0.42V	(2e⁻)
NO_3^-/ ½ N_2	+0.74V	(5e⁻)
Fe^{3+}/Fe^{2+} (pH 2)	+0.76V	(1e⁻)
½ O_2/H_2O	+0.82V	(2e⁻)

5.1 Electron Tower ♦ Redox pairs are listed from top to bottom in order of decreasing reduction potential (not to scale). The more positive the charge is, the lower the reduction potential. The oxidant is on the left; the reductant is on the right. Electrons are transferred downward from the reductant of one pair to the oxidant of a pair containing lower reduction potential. The actual reduction potentials of each pair is in red. Also included are the numbers of electrons (in parentheses) transferred in any one reaction.

[1] The formula for calculating the energy released (or required) in any given redox reaction in the tower is $\Delta G^{0'} = -nF\Delta E_0'$, where $\Delta G^{0'}$ is the change in energy in kilojoules (at standard temperature and pressure), n is the number of electrons in the transfer, F is the Faraday constant (96.48 kilojoules/volt), and E_0' is the difference (in volts) of the two chemicals' reduction potentials. For example, calculation of the two-electron transfer from the $2H^+/H_2$ redox pair (reduction potential = +0.41 volts) to the $½O_2/H_2O$ pair (reduction potential = +0.82 volts) would be thus: $\Delta G^{0'} = -nF\Delta E_0' = -237.34$ kj. By convention, negative $\Delta G^{0'}$ symbolizes an exergonic reaction yielding free energy. Conversely, calculating the reaction in reverse reveals a positive $\Delta G^{0'}$ and, a 237.34 kj input requirement.

As shown in the tower, chemicals are presented as redox pairs with the oxidant on the left and the reductant on the right separated by a slash, as in $2H^+/H_2$ and Fe^{3+}/Fe^{2+}. The pairs are listed in descending order with molecules containing the highest reduction potential at the top. Therefore, all spontaneous transfers (without energy input) as shown in the tower are downward. A typical transfer (from reductant to oxidant) would be $H_2 + \frac{1}{2}O_2 \rightarrow H_2O$.

Note: Redox pairs higher in the tower have a negative reduction potential ($H^+/H_2 = -0.50$ volts), while pairs lower in the tower have a positive reduction potential ($NO_3^-/NO_2^- = +0.42$). This may seem to be a contradiction, given that the molecules higher in the tower have greater reduction potential, but the numbers are actual voltages related to the charges and stability of the molecules. Therefore, the more negative a chemical's charge, the greater its reduction potential, and the more likely it will be to donate electrons.

Although transfers tend to be from the molecules at or near the top, any molecule theoretically could transfer electrons to a molecule with a lower reduction potential. The amount of energy released in any one transfer is dependent on the difference between the reduction potentials of the two molecules and the number of electrons transferred. Relatively speaking, two molecules that are far apart on the tower will release greater energy than two molecules that are closer together. Therefore, even a quick glance at the tower, without knowing the reduction potentials of the molecules involved, tells you that the reduction of NO_3 by H_2 would produce more free energy than the reduction of Fe^{3+} by H_2.

Although the formula is included for those of you who are interested in exploring the subject further, today's exercise does not involve energy calculations. Instead, you will make a simple qualitative examination of the physical consequences of free energy release.

The organism chosen for this exercise, *Proteus mirabilis*, is a respirer that preferentially will reduce oxygen at the end of the electron transport chain (for examples, see Fig. 5.19), but can reduce other inorganic molecules if oxygen is not available. In liquid media, oxygen can be made unavailable by placing a layer of mineral oil on top of the broth, which cuts off oxygen diffusion from the air, thereby creating an anaerobic environment where alternate electron acceptors must be used.

The alternate electron acceptor used in this exercise is thiosulfate ($S_2O_3^{2-}$). As you examine the electron tower, note the location of the $2H^+/H_2$, $\frac{1}{2}O_2/H_2O$ and $S_2O_3^{2-}/HS^-$ redox pairs. Because the difference between the $2H^+/H_2 - \frac{1}{2}O_2/H_2O$ reduction potentials is greater than the difference between the $2H^+/H_2 - S_2O_3^{2-}/HS^-$ reduction potentials, a reaction involving the former would be expected to yield more energy than a reaction involving the latter. Therefore, the presumption (your hypothesis) is that the pathway yielding greater energy (and more ATP) will produce greater growth than the pathway yielding lower energy.

Application

This exercise is for demonstration purposes only.

In This Exercise

Today, you will begin growing an organism that is capable of both aerobic and anaerobic respiration. You will inoculate two different growth media in four flasks. One of each pair of broths will be covered with mineral oil to prevent diffusion of oxygen into the medium and promote anaerobic growth. The test organism—*Proteus mirabilis*—is capable of reducing either oxygen or thiosulfate, so sodium thiosulfate was added as the alternate electron acceptor to one of the media. After a day or two, you will compare the density of growth in all broths, record your observations, and see if your hypothesis is supported.

✓ Materials

Per Student Group

- ☐ Four 25 mL Erlenmeyer flasks containing nutrient broth[2]
- ☐ Four 25 mL Erlenmeyer flasks containing nutrient broth + thiosulfate
- ☐ One sterile Pasteur or disposable pipette with appropriate bulb
- ☐ Sterile mineral oil
- ☐ Four sterile transfer pipettes
- ☐ Gloves
- ☐ Eye protection
- ☐ Fresh broth culture of:
  - *Proteus mirabilis* (BSL-2)

Media Recipes

Nutrient Broth

• Beef extract	3.0 g
• Peptone	5.0 g
• Distilled or deionized water	1.0 L

[2] Test tubes may be substituted for flasks with proportionately less medium in each.

Nutrient Broth with Thiosulfate

• Beef extract	3.0 g
• Peptone	5.0 g
• Sodium thiosulfate	0.3 g
• Distilled or deionized water	1.0 L

Procedure

Lab One

1. *P. mirabilis* is a BSL-2 organism. **Be sure to employ BSL-2 precautions throughout this exercise.**
2. Obtain all materials needed for the exercise.
3. Label two of the nutrient broth flasks "NB" and two flasks "NB Control."
4. Label the other four flasks similarly as "NBT" and "NBT Control."
5. Using a different sterile transfer pipette for each, inoculate the two NB and two NBT flasks. Do not inoculate the control flasks.
6. As shown in Figure 5.2, aseptically add 1 mL–2 mL sterile mineral oil to one NB, one NBT, and one of each control flask (Fig. 5.3).
7. Incubate all flasks at 35 ± 2°C until the next lab period.
8. Write your predictions for relative amounts of growth in the table provided on the data sheet, page 293 under question 1. Record them in decreasing order of turbidity.

Lab Two

1. Remove all flasks from the incubator and examine them for growth.
2. Record your observations in the table on the data sheet. Use a scale of "0" for no turbidity through "+ + + +" for heavy turbidity.

5.2 Adding Mineral Oil ◆ Aseptically add sterile mineral oil. Be sure to tilt the flask and add the oil slowly to avoid creating bubbles.

5.3 Aerobic vs Anaerobic ◆ Cover one broth in each pair of flasks with oil to promote anaerobic growth.

Reference

Zimbro, Mary Jo and David A. Power, eds. Page 408 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-1

Reduction Potential

OBSERVATIONS AND INTERPRETATIONS

1 Record your results and interpretations in the table below. Use a scale of "0" for no turbidity through "+ + + +" for heavy turbidity. Your interpretation should include the likely type of metabolism performed in each flask.

Flask Contents	Amount of Growth/Turbidity		Interpretation
	P. mirabilis	Control	
Nutrient broth			
Nutrient broth with oil			
Nutrient broth + thiosulfate			
Nutrient broth + thiosulfate with oil			

QUESTIONS

1 *List your predicted results and your actual results in the following table in descending order of turbidity. Defend your prediction and offer possible reasons for any disagreements between the expected and actual results.*

Expected Result	Actual Result

2 *Consider the controls.*

a. *What purpose do the controls serve in this experiment? Explain your answer.*

b. *What is the desired result in each control?*

c. *How many and what combinations of results are acceptable?*

3 *Using Figure 5.1 as a reference (without regard to equation balance), circle all of the following reactions that will go forward without the addition of energy.*

$H_2 + \frac{1}{2}O_2 \rightarrow H_2O$

$Fe^{2+} + SO_4^{2-} \rightarrow FeSO_4$

$H_2 + NO_3^- \rightarrow NO_2^- + H_2O$

cytochrome a_{red} + cytochrome c_{ox} → cytochrome a_{ox} + cytochrome c_{red}

EXERCISE
5-2

Oxidation–Fermentation Test

Theory

The Oxidation–Fermentation (O–F) test is designed to differentiate bacteria on the basis of fermentative or oxidative metabolism of carbohydrates. In this medium, oxidative organisms oxidize the carbohydrate to CO_2, H_2O, and energy, using in the following order: glycolysis, the oxidation of pyruvate, the citric acid cycle, and finally the electron transport chain (ETC) with oxygen (the final electron acceptor, FEA) being reduced to H_2O. Similarly, fermentative organisms convert the carbohydrate to pyruvate, but because either they *cannot* use O_2 as an FEA or it is *not available*, pyruvate, or one of its derivatives, acts as the FEA and it is reduced to the familiar acid, gas, and/or alcohol fermentation end products. Consequently, fermenters identified by this test acidify O–F medium to a greater extent than do oxidizers.

Hugh and Leifson's O–F medium includes a high sugar-to-peptone ratio to reduce the possibility that alkaline products from peptone utilization will neutralize weak acids produced by oxidation of the carbohydrate. Bromothymol blue dye, which is yellow at pH 6.0, green at pH 7.1, and blue at pH 7.6, is added as the indicator. A low agar concentration makes it a semisolid medium that also allows determination of motility.

The medium is prepared with glucose, lactose, sucrose, maltose, mannitol, or xylose and is not slanted. Two tubes of the specific sugar medium are stab inoculated several times with the test organism. After inoculation, one tube is sealed with a layer of sterile mineral oil to promote anaerobic growth and fermentation (Fig. 5.4). The mineral oil creates an environment unsuitable for oxidation because it prevents diffusion of oxygen from the air into the medium. The other tube is left unsealed to allow aerobic growth and oxidation. (*Note*: Tubes of O–F medium are heated in boiling water and then cooled prior to inoculation. This removes free oxygen from the medium and ensures an anaerobic environment in all tubes. The tubes covered with oil will remain anaerobic, whereas the uncovered medium quickly will become aerobic as oxygen diffuses back in.) O-F Test results are summarized in Table 5-1 and shown in Figure 5.5.

Organisms that are able to ferment the carbohydrate or ferment *and* oxidize the carbohydrate will turn the sealed and unsealed media yellow throughout, as will organisms that are able to ferment only. Organisms that are able to oxidize only will turn the unsealed medium yellow (or partially yellow) and leave the sealed medium green or blue. Slow or weak fermenters will turn both tubes slightly yellow at the top. Organisms that are not able to metabolize the sugar will either produce no color change or turn the medium blue because of alkaline products from amino acid degradation (deamination).

5.4 Adding the Sterile Mineral Oil Layer ♦ Tip the tube slightly to one side, and gently add 3 mm–4 mm of sterile mineral oil. Be sure to use a sterile pipette for each tube.

Application

The O–F test is used to differentiate bacteria based on their ability to oxidize or ferment specific sugars. It allows presumptive separation of the fermentative *Enterobacteriaceae* from the oxidative *Pseudomonas* and *Bordetella*, and the nonreactive *Alcaligenes* and *Moraxella*.

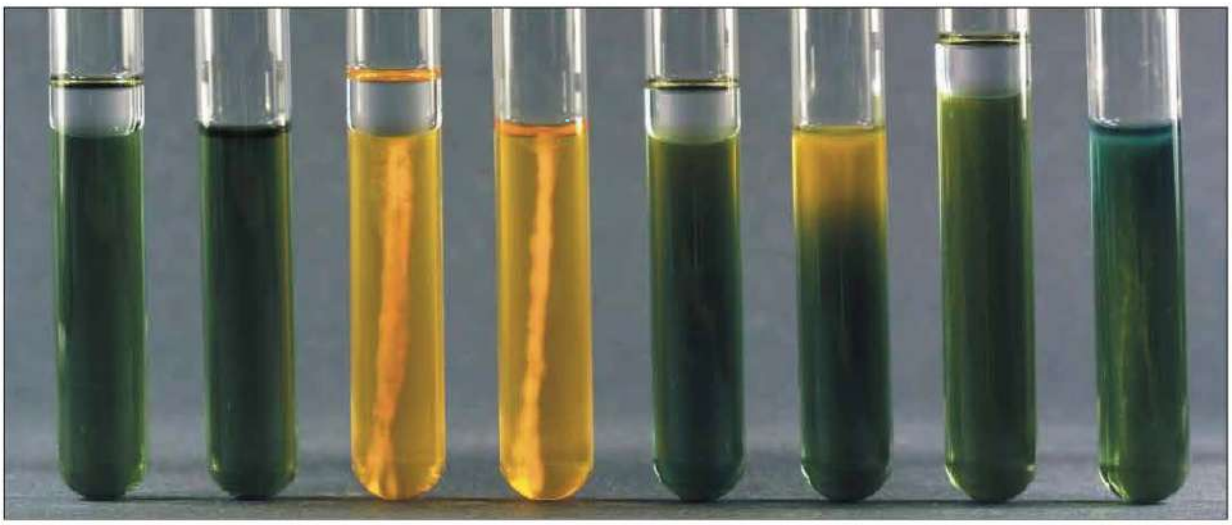
In This Exercise

Eight O–F media tubes will be used for this exercise—six for inoculation (two for each organism) and two for controls. One of each pair will receive a mineral oil overlay to maintain an anaerobic environment and force fermentation if the organism is capable of doing so. You will not do a motility determination, because motility is covered in Exercise 5-28. For best results, use a heavy inoculum and several stabs for each tube. Use only sterile pipettes to add the mineral oil.

TABLE 5-1 O–F Medium Results and Interpretations

Table of Results			
Sealed	**Unsealed**	**Interpretation**	**Symbol**
Green or blue	Any amount of yellow	Oxidation	O
Yellow throughout	Yellow throughout	Oxidation and fermentation or fermentation only	O–F or F[1]
Slightly yellow at the top	Slightly yellow at the top	Oxidation and slow fermentation or slow fermentation only	O–F or F[1]
Green or blue	Green or blue	No sugar metabolism; organism is nonsaccharolytic	N

[1] The results of a fermentative organism and one capable of both fermentative and oxidative metabolism (of that specific carbohydrate) look the same in this medium. Therefore, when both tubes are yellow, the result is recorded as (O-F) or (F)



5.5 Oxidation–Fermentation Test Results ◆ These pairs of tubes illustrate three possible results in the O–F test. Reading from left to right, pair 1 was uninoculated for color comparison. Pair 2 was inoculated with an organism capable of both oxidative and fermentative utilization of glucose (O–F). Pair 3 was inoculated with an organism capable only of oxidative metabolism (O). Pair 4 was inoculated with an organism incapable of utilizing glucose. Note the blue color in the upper portion of the unsealed tube suggesting that the organism is both nonsaccharolytic (N) and strictly aerobic.

✓ Materials

Per Student Group

- ☐ Eight O–F glucose tubes
- ☐ Sterile mineral oil
- ☐ Sterile transfer pipettes
- ☐ Fresh agar slants of:
  - *Alcaligenes faecalis*
  - *Escherichia coli*
  - *Kocuria rosea*

✓ Medium Recipe

Hugh and Leifson's O–F Medium with Glucose

• Pancreatic digest of casein	2.0 g
• Sodium chloride	5.0 g
• Dipotassium phosphate	0.3 g
• Agar	2.5 g
• Bromothymol blue	0.08 g
• Glucose	10.0 g
• Distilled or deionized water	1.0 L

pH = 6.6–7.0 at 25°C

Procedure

Lab One

1. Obtain eight O–F tubes. Label six (in three pairs) with the names of the organisms, your name, and the date. Label the last pair "control."
2. Using an inoculating needle, stab inoculate each pair with the appropriate test organism. Stab several times to a depth of about 1 cm from the bottom of the agar to produce a heavy inoculum. Do not inoculate the controls.
3. Using a different sterile transfer pipette for each organism, overlay one of each pair of tubes (including the controls) with 3 mm–4 mm of sterile mineral oil (Fig. 5.4).
4. Incubate all tubes at 35 ± 2°C for 48 hours.

Lab Two

1. Examine the tubes for color changes. Be sure to compare them to the controls before making a determination.
2. Record your results in the table provided on the data sheet, page 299.

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 112 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Delost, Maria Dannessa. Pages 218–219 in *Introduction to Diagnostic Microbiology.* St. Louis: Mosby, 1997.

MacFaddin, Jean F. Page 379 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Smibert, Robert M. and Noel R. Krieg. Page 625 in *Methods for General and Molecular Bacteriology.* Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Tille, Patricia M. Pages 98 and 224 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis: Mosby, 2014.

Zimbro, Mary Jo and David A. Power, eds. Page 410 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media.* Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-2

Oxidation-Fermentation Test

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 5-1, page 296, when recording your results and interpretations in the table below.

Organism	Color Results		Symbol	Interpretation
	Sealed	Unsealed		
Uninoculated control				

QUESTIONS

1 *Consider the controls.*

a. *What is the purpose of the uninoculated control tubes used in this test?*

b. *Are the controls positive or negative controls? Explain.*

c. *Why is it necessary to use two controls rather than just one? What are the specific purposes of each? Be thorough in your answer.*

2 *Some microbiologists recommend inoculating a pair of O–F basal media (without carbohydrate) along with the carbohydrate media. Why do you think this is done?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-2

(continued)

3 *All enterics* (Enterobacteriaceae) *are facultative anaerobes; that is, they have both respiratory and fermentative enzymes. What color results would you expect for organisms in O–F glucose media inoculated with an enteric? Remember to describe both sealed and unsealed tubes.*

4 *Suppose that when you examined your tubes (in this exercise) after incubating them, you noticed that the unsealed control contained slight yellowing at the top. Suppose further that pair 1 showed complete yellowing of both tubes and pairs 2 and 3 showed slight yellowing of the unsealed tube. Assuming all other tubes were green, what conclusions could you safely make?*

Which results, if any, are reliable? Why?

Which results, if any, are not reliable? Why not?

5 *Uninoculated tubes and tubes inoculated with an organism that is (N) serve as negative controls. What information is provided by each?*

Fermentation Tests

As defined at the beginning of this section, carbohydrate fermentation is the metabolic process by which an organic molecule acts as an electron donor (becoming oxidized in the process) and one or more of its organic products act as the final electron acceptor (FEA). In actuality, the term **carbohydrate fermentation** is used rather broadly to include hydrolysis of disaccharides prior to the fermentation reaction. Thus, a "lactose fermenter" is an organism that splits the disaccharide lactose into the monosaccharides glucose and galactose and then ferments the monosaccharides. In this section you will see the term **fermenter** frequently. Unless it is expressly used otherwise, this term should be assumed to include the initial hydrolysis and/or conversion reactions.

Fermentation of glucose begins with the production of pyruvate. Although some organisms use alternative pathways (e.g., Entner-Doudoroff pathway, Fig. A.2) most bacteria accomplish this by glycolysis. The end products of pyruvate fermentation include a variety of organic acids, alcohols, and hydrogen or carbon dioxide gas. The specific end products depend on the specific organism and the substrate fermented. Refer to Figures 5.6 and A.5.

In this unit you will perform three tests that identify different fermentation abilities. Two require the use of differential fermentation media—phenol red (PR) broth and MR-VP broth. The other is a rapid test that determines the presence of β-galactosidase within a couple of hours and doesn't require the standard 24-48 hours of incubation. Phenol red broth is a general-purpose fermentation medium. Typically, it contains any one of several carbohydrates (e.g., glucose, lactose, sucrose), a pH indicator, and a Durham tube to detect acid and gas formation, respectively. MR-VP broth is a dual-purpose medium that tests an organism's ability to follow either (or both) of two specific fermentation pathways. The methyl red (MR) test detects what is called a **mixed acid fermentation**. The Voges-Proskauer (VP) test identifies bacteria that are able to produce acetoin as part of a **2,3-butanediol fermentation**. The ONPG test allows quick differentiation between organisms that can't ferment lactose (because they lack β-galactosidase) from those that can, but do so slowly because of difficulty transporting lactose into the cell. See Appendix A, Oxidation of Pyruvate (page 830), for more information about fermentation. ◆

EXERCISE
5-3
Phenol Red Broth

Theory

Phenol red (PR) broth is a differential test medium prepared as a base to which a carbohydrate is added. Included in the base medium are peptone and the pH indicator phenol red. Phenol red is yellow below pH 6.8, pink to magenta above pH 7.4, and red in between. During preparation the pH is adjusted to approximately 7.3 so it appears red. Finally, an inverted Durham tube is added to each tube as an indicator of gas production.

Acid production from fermentation of the carbohydrate lowers the pH below the neutral range of the indicator and turns the medium yellow (Fig. 5.7). Deamination of amino acids supplied by peptone results in an alkaline reaction from the ammonia (NH_3) that is produced, which raises the pH and turns the broth pink. Gas production, also from fermentation, is indicated by a bubble, or pocket, in the Durham tube where the broth has been displaced.

Application

PR broth is used to differentiate members of *Enterobacteriaceae* and to distinguish them from other Gram-negative rods. It also is used to distinguish between Gram-positive fermenters, such as *Streptococcus* and *Lactobacillus* species.

In This Exercise

Today you will inoculate PR broths with four organisms to determine their fermentation characteristics. Use Table 5-2 as a guide when interpreting and recording your results. Be sure to use an uninoculated control of each medium for color comparison.

✓ Materials

Per Student Group

- ☐ Five PR glucose broths with Durham tubes
- ☐ Five PR lactose broths with Durham tubes
- ☐ Five PR sucrose broths with Durham tubes
- ☐ Five PR base broths with Durham tubes
- ☐ Fresh cultures of:
  - *Alcaligenes faecalis*
  - *Escherichia coli*
  - *Providencia stuartii*
  - *Streptococcus gallolyticus*

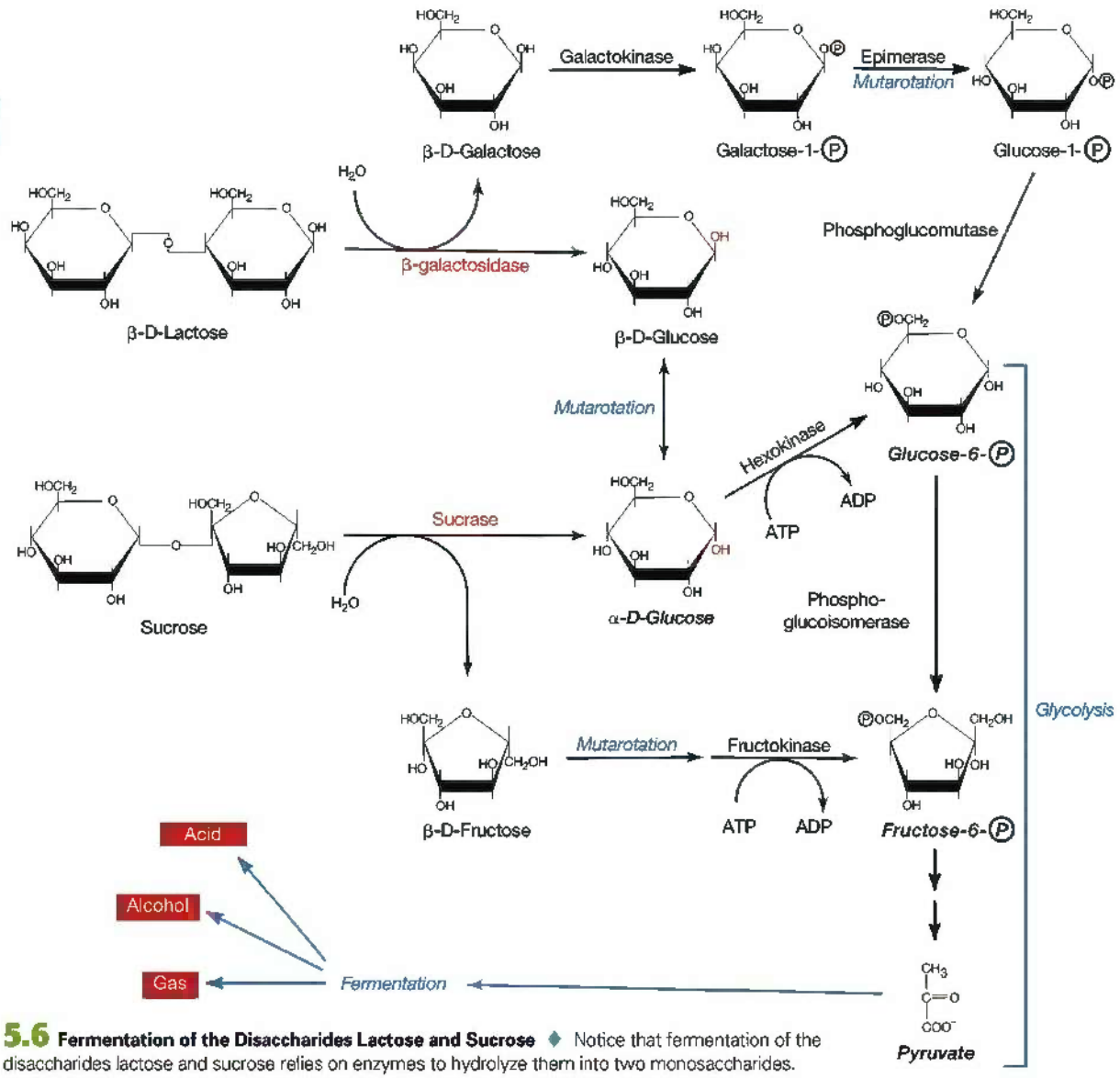
■ Medium Recipe

PR (Carbohydrate) Broth[1]

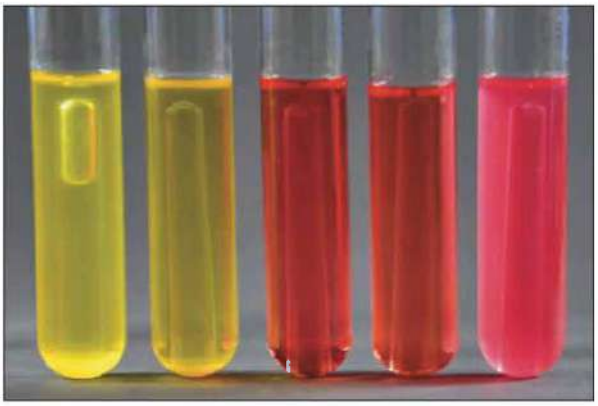
• Pancreatic digest of casein	10.0 g
• Sodium chloride	5.0 g
• Carbohydrate (glucose, lactose, sucrose)	5.0 g
• Phenol red	0.018 g
• Distilled or deionized water	1.0 L

pH = 7.1–7.5 at 25°C

[1] This formulation without carbohydrate is PR base broth.



5.6 Fermentation of the Disaccharides Lactose and Sucrose ♦ Notice that fermentation of the disaccharides lactose and sucrose relies on enzymes to hydrolyze them into two monosaccharides. Once the monosaccharides are formed, they are fermented via glycolysis, just as glucose is.



5.7 PR Glucose Broth Results ♦ From left to right, these five PR glucose broths demonstrate acid and gas production (A/G), acid production without gas (A/–), uninoculated control tube for comparison, no reaction (–/–), and alkaline reaction as a result of peptone degradation (K).

TABLE 5-2 Phenol Red (PR) Broth Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Yellow broth, bubble in tube	Fermentation with acid and gas end products	A/G
Yellow broth, no bubble in tube	Fermentation with acid end products; no gas produced	A/–
Red broth, no bubble in tube	No fermentation	–/–
Pink broth, no bubble in tube	Degradation of peptone; alkaline end products	K

Procedure

Lab One

1. Obtain five of each PR broth. Label each with the name of the organism, your name, the medium name, and the date. Label the fifth tube of each medium "control."
2. Inoculate one of each broth with each test organism.
3. Incubate all of the tubes at 35 ± 2°C for 48 hours.

Lab Two

Using the uninoculated controls for color comparison and Table 5-2 as a guide, examine all of the tubes and enter your results in the table provided on the data sheet, page 307. When indicating the various reactions, use the standard symbols as shown, with the acid reading first, followed by a slash and then the gas reading. K indicates alkalinity and –/– symbolizes no reaction. *Note*: Do not try to read these results without the appropriate control tubes for comparison.

References

Lányi, B. Page 44 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 57 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Zimbro, Mary Jo and David A. Power, eds. Page 440 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-3

Phenol Red Broth

OBSERVATIONS AND INTERPRETATIONS

1 Enter your results (color and bubble/no bubble) in the table below, using the symbols shown in Table 5-2, page 305. Be sure to include the relevant enzyme's name in your interpretation if an organism ferments sucrose and/or lactose.

Organism	Results				Interpretation
	PR Base	PR Glucose	PR Lactose	PR Sucrose	
Uninoculated control					GLU
					LAC
					SUC
					GLU
					LAC
					SUC
					GLU
					LAC
					SUC
					GLU
					LAC
					SUC
					GLU
					LAC
					SUC

QUESTIONS

1 *Consider the controls.*

a. *Were the uninoculated controls positive or negative controls, and what purpose did they serve?*

b. *What purpose did the PR base broths serve?*

2 *Early formulations of this medium used a smaller amount of carbohydrate and occasionally produced false (pink) results after 48 hours. This phenomenon is called a reversion.*

a. *Why do you think this happened?*

b. *List at least two steps, as a microbiologist, you could take to prevent the problem.*

3 *Suppose you inoculate a PR broth with an organism* known to be a slow-growing fermenter. *After 48 hours, you see slight turbidity but score it as (−/−).*

a. *Is this result a false positive or a false negative?*

b. *Is this false result caused by poor specificity or poor sensitivity of the test system?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-3

(continued)

4 *Assuming you tested an organism using the three carbohydrate broths and a base broth, which of the combinations of results in the following table would be reliable? Interpret each of the combinations and explain why the results are reliable or not. Assume all tubes in a series were inoculated with the same amount of organism and were incubated for 24 hours.*

PR Base	PR Glucose	PR Lactose	PR Sucrose	Interpretation	Reliable? Y/N
–/–	A/G	A/G	A/G		
–/–	A/–	A/–	–/–		
A/G	A/G	A/G	A/G		
–/–	A/G	A/G	K		
K	K	K	K		
–/–	K	A/–	–/–		
K	A/G	A/G	–/–		
A/–	–/–	–/–	–/–		

Methyl Red and Voges–Proskauer Tests

EXERCISE 5-4

Theory

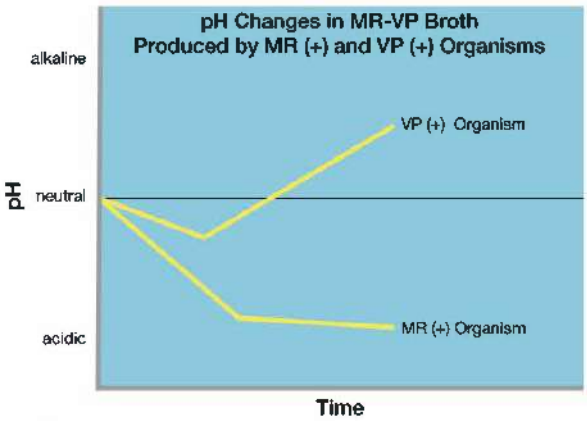
Methyl red and Voges-Proskauer (MR-VP) broth is a combination medium used for both methyl red (MR) *and* Voges-Proskauer (VP) tests. It is a simple solution containing only peptone, glucose, and a phosphate buffer. The peptone and glucose provide protein (with its nitrogen) and fermentable carbohydrate, respectively, and the potassium phosphate resists pH changes in the medium.

The MR test is designed to detect organisms capable of performing **a mixed acid fermentation**, which overcomes the phosphate buffer in the medium and lowers the pH (Figs. 5.8 and 5.9). The acids produced by these organisms tend to be stable, whereas acids produced by other organisms tend to be unstable and subsequently are converted to more neutral products.

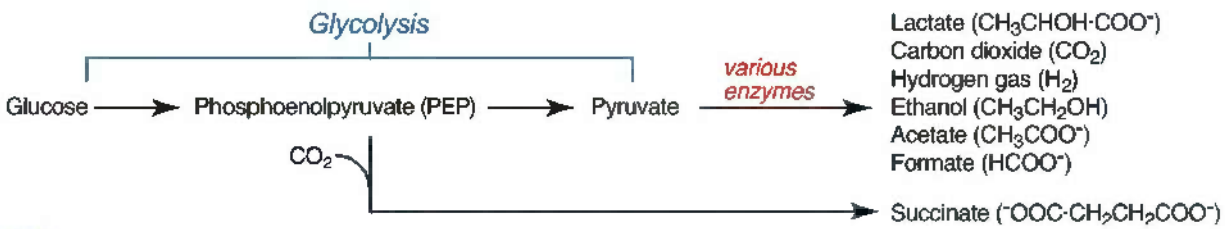
Mixed acid fermentation is verified by the addition of methyl red indicator dye following incubation. Methyl red is red at pH 4.4 and yellow at pH 6.2. Between these two pH values, it is various shades of orange. Red color is the only true indication of a positive result. Orange is negative or inconclusive. Yellow is negative (Fig. 5.10).

The Voges-Proskauer test was designed for organisms that are able to ferment glucose, but quickly convert their acid products to acetoin and 2,3-butanediol (Figs. 5.9 and 5.11). Adding VP reagents to the medium oxidizes the **acetoin** to **diacetyl**, which in turn reacts with **guanidine nuclei** from peptone to produce a red color (Fig. 5.12). A positive VP result, therefore, is red. No color change (or development of copper color) after the addition of reagents is negative. The copper color is a result of interactions between the reagents and should not be confused with the true red color of a positive result (Fig. 5.13). Use of positive and negative controls for comparison is usually recommended.

To avoid conflicting results of the two tests, two 1 mL volumes are transferred from the MR-VP broth to separate test tubes after incubation. Methyl red indicator reagent is added to one tube, and VP reagents are added to the other. Color changes then are observed and documented. Refer to the procedural diagram in Figure 5.14.



5.9 pH Changes in MR-VP Broth ♦ The MR test identifies organisms that perform a mixed acid fermentation and produce stable acid end products. MR (+) organisms lower the broth's pH permanently. The VP test is used to identify organisms that perform a 2,3-butanediol fermentation. VP (−) organisms initially may produce acid and temporarily lower the pH, but because the 2,3-butanediol fermentation end products are neutral, the pH at completion of the test is near neutral.



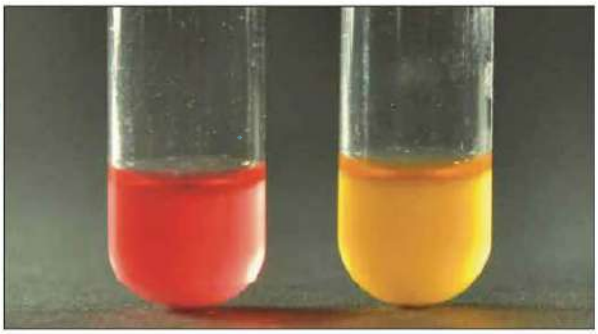
5.8 Mixed Acid Fermentation of *E. coli* ♦ *E. coli* is a representative methyl red positive organism and is recommended as a positive control for the test. Its mixed acid fermentation produces the end products listed in order of abundance. Most of the formate is converted to H_2 and CO_2 gases. ***Note:*** The amount of succinate falls between acetate and formate but is derived from PEP, not pyruvate. *Salmonella* and *Shigella* also are methyl red positive.

Application

The methyl red and Voges-Proskauer tests are components of the *IMViC* battery of tests (*I*ndole, *M*ethyl red, *V*oges-Proskauer, and *C*itrate) used to distinguish between members of the family *Enterobacteriaceae* and differentiate them from other Gram-negative rods. For more information about these fermentations, refer to Figure A.5.

In This Exercise

Today you will perform methyl red and Voges-Proskauer tests on *Escherichia coli* and *Enterobacter aerogenes*. After a 48-hour incubation period, you will observe the results of a mixed acid fermentation and a 2,3-butanediol fermentation. The exercise involves transfer of a portion of broth containing living organisms and the measured addition of reagents to complete the observable reactions. Please use care in your transfers and in your measurements. For help with the procedure, refer to Figure 5.14. For help interpreting your results, refer to Figures 5.10 and 5.13 and Tables 5-3 and 5-4.



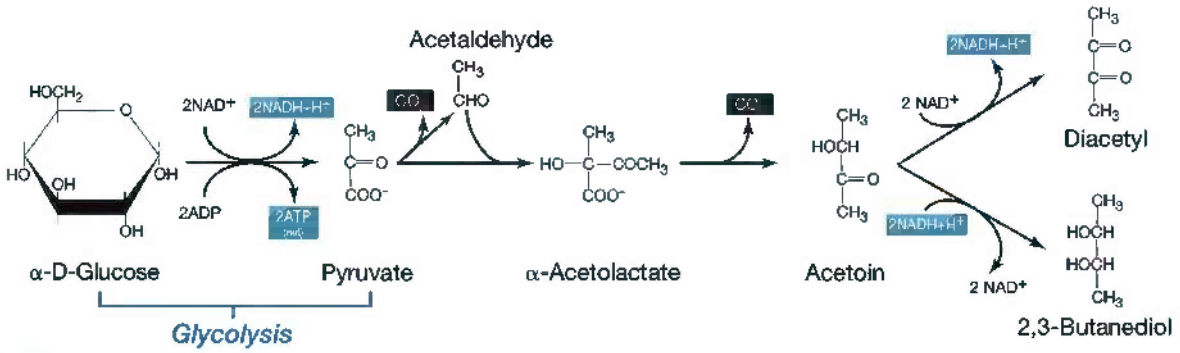
5.10 Methyl Red Test Results ◆ The tube on the left is MR positive. The tube on the right is MR negative.

TABLE 5-3 Methyl Red Test Results and Interpretations

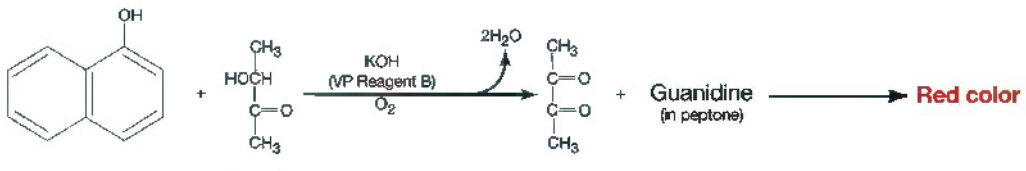
Table of Results		
Result	**Interpretation**	**Symbol**
Red	Mixed acid fermentation	+
No color change	No mixed acid fermentation	–

TABLE 5-4 Voges-Proskauer Test Results and Interpretations

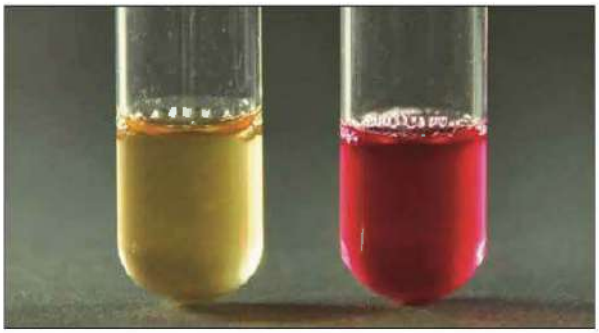
Table of Results		
Result	**Interpretation**	**Symbol**
Red	2,3-butanediol fermentation (acetoin produced)	+
No color change	2,3-butanediol fermentation (acetoin is not produced)	–



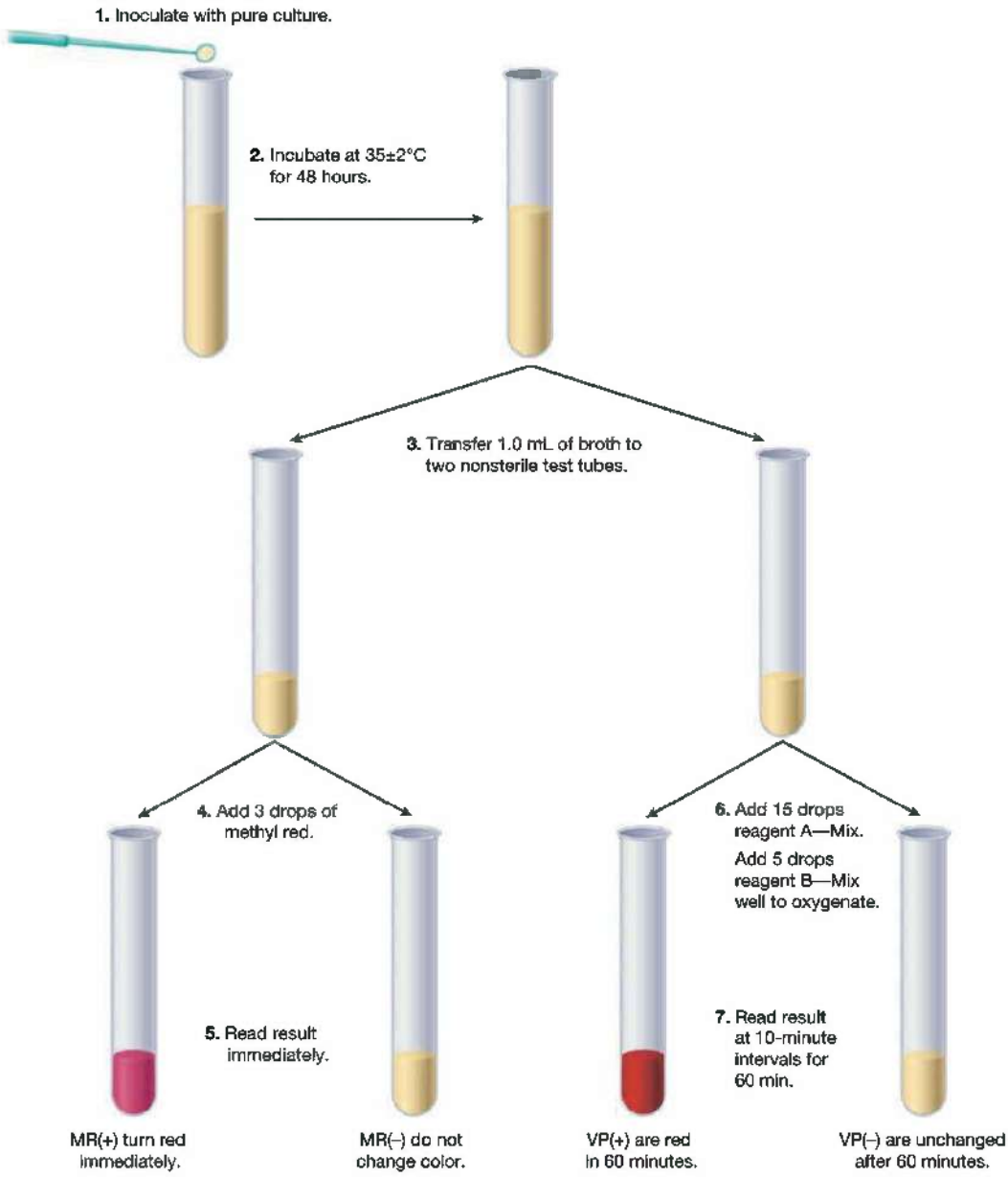
5.11 2,3-Butanediol Fermentation ◆ Acetoin is an intermediate in this fermentation. Reduction of acetoin by NADH leads to the end product 2,3-butanediol. Oxidation of acetoin produces diacetyl, as in the indicator reaction for the VP test (see Fig. 5.12).



5.12 Indicator Reaction of Voges-Proskauer Test ◆ Reagents A and B are added to VP broth after 48 hours of incubation. These reagents react with acetoin and oxidize it to diacetyl, which in turn reacts with guanidine (from the peptone in the medium) to produce a red color.



5.13 Voges-Proskauer Test Results ♦ The tube on the left is VP negative. The tube on the right is VP positive.


1. Inoculate with pure culture.
2. Incubate at 35±2°C for 48 hours.
3. Transfer 1.0 mL of broth to two nonsterile test tubes.
4. Add 3 drops of methyl red.
5. Read result immediately.
6. Add 15 drops reagent A—Mix.
Add 5 drops reagent B—Mix well to oxygenate.
7. Read result at 10-minute intervals for 60 min.
MR(+) turn red immediately.
MR(–) do not change color.
VP(+) are red in 60 minutes.
VP(–) are unchanged after 60 minutes.

5.14 Procedural Diagram for the Methyl Red and Voges-Proskauer Tests ♦ Be sure to wear gloves and chemical eye protection when adding reagents, mixing, and reading results.

✓ Materials

Per Student

- □ Gloves
- □ Chemical eye protection

Per Student Group

- □ Three MR-VP broths
- □ Methyl red reagent
- □ VP reagents A and B
- □ Six nonsterile test tubes
- □ Three nonsterile 1 mL pipettes
- □ Fresh cultures of:
  - *Escherichia coli*
  - *Enterobacter aerogenes*

■ Medium and Reagent Recipes

5

MR-VP Broth

• Buffered peptone	7.0 g
• Dipotassium phosphate	5.0 g
• Dextrose (glucose)	5.0 g
• Distilled or deionized water	1.0 L
pH 6.7–7.1 at 25°C	

Methyl Red Reagent

• Methyl red dye	0.1 g
• Ethanol	300.0 mL
• Distilled water to bring volume to	500.0 mL

VP Reagent A

• α-naphthol	5.0 g
• Absolute alcohol to bring volume to	100.0 mL

VP Reagent B

• Potassium hydroxide	40.0 g
• Distilled water to bring volume to	100.0 mL

Procedure

Lab One

1. Obtain three MR-VP broths. Label two of them with the names of the organisms, your name, and the date. Label the third tube "control."
2. Inoculate two broths with the test cultures. Do not inoculate the control.
3. Incubate all tubes at 35 ± 2°C for 48 hours.

Lab Two

1. Wear gloves and chemical eye protection for this part of the procedure.
2. Mix each broth well. Transfer 1.0 mL from each broth to two nonsterile tubes. The pipettes should be sterile, but the tubes need not be. Refer to the procedural diagram in Figure 5.14.
3. For the three pairs of tubes, add the reagents as follows.

Tube #1: VP Test

a. Add 15 drops (0.6 mL) of VP reagent A. Mix well to oxygenate the medium, but be careful not to splash it.

b. Add 5 drops (0.2 mL) of VP reagent B. Carefully mix well again to oxygenate the medium.

c. Place the tubes in a test tube rack and observe for red color formation at regular intervals starting at 10 minutes for up to 1 hour. Mix again periodically to oxygenate the medium.

d. Using Table 5-4 as a guide, record your results in the table provided on the data sheet, page 315. Sometimes it is difficult to differentiate weak positive reactions from negative reactions. VP (−) reactions often produce a copper color. Weak VP (+) reactions produce a pink color. The strongest color change should be at the surface.

Tube #2: Methyl Red Test

a. Add three drops of methyl red reagent. Observe for red color formation immediately.

b. Using Table 5-3 as a guide, record your results in the table provided on the data sheet.

References

Chapin, Kimberle C. and Tsai-Ling Lauderdale. Page 361 in *Manual of Clinical Microbiology,* 8th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Michael A. Pfaller, and Robert H. Yolken, eds. Washington, DC: ASM Press, American Society for Microbiology, 2003.

Delost, Maria Dannessa. Pages 187–188 in *Introduction to Diagnostic Microbiology.* St. Louis, MO: Mosby, 1997.

MacFaddin, Jean F. Pages 321 and 439 in *Biochemical Tests for Identification of Medical Bacteria,* 3rd ed. Baltimore: Williams & Wilkins, 2000.

Tille, Patricia M. Page 214 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power, eds. Page 330 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media.* Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-4

Methyl Red and Voges–Proskauer Tests

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Tables 5-3 and 5-4, page 312, as you record your results (color) and interpretations in the table below.

Organism	MR Result	VP Result	Interpretation
Uninoculated control			

QUESTIONS

1 *Some protocols call for a shorter incubation time for the MR and VP tests. Other protocols allow for up to 10 days incubation with virtually no risk of producing a false positive.*

a. *Which of the two tests would likely produce more false negatives with a shorter incubation time? Why?*

_____

_____

_____

_____

_____

b. *Which test would likely benefit most from a longer incubation time? Why?*

_____

_____

_____

_____

_____

2 *Would a false negative result for the VP test more likely be attributable to poor sensitivity or to poor specificity of the test system?*

3 *Why were you told to shake the VP tubes after the reagents were added?*

4 *Why is the methyl red test read immediately after addition of methyl red reagent and the Voges-Proskauer read up to 60 minutes after addition of VP reagents A and B?*

5 *Some microbiologists recommend reincubating organisms producing methyl red negative results for an additional 2 to 3 days. Why do you think this is done?*

ONPG Test

EXERCISE 5-5

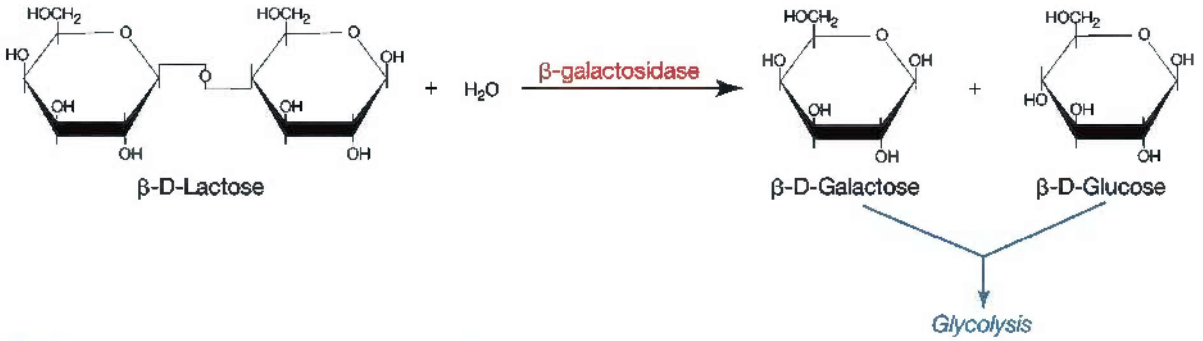
Theory

Bacteria that ferment lactose typically produce two enzymes: **β-galactoside permease**, a membrane-bound transport protein; and **β-galactosidase**, an intracellular enzyme that hydrolyzes the disaccharide into β-glucose and β-galactose (Fig. 5.15).

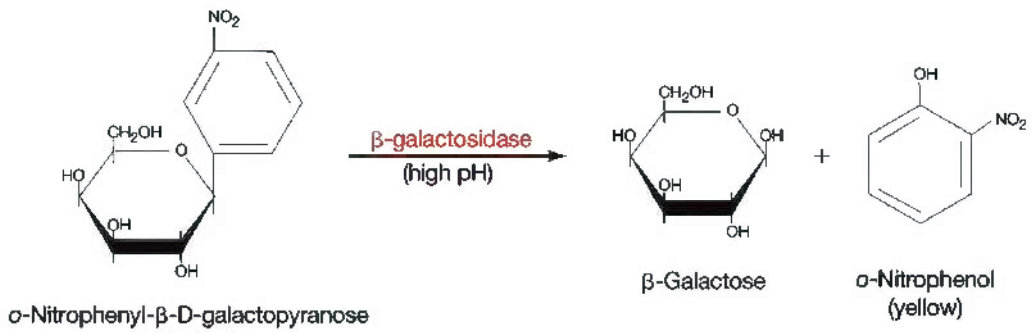
Bacteria possessing both enzymes are active β-lactose fermenters. Bacteria that cannot produce β-galactosidase cannot ferment β-lactose. Bacteria that possess β-galactosidase but no (or small amounts of) β-galactoside permease are slow to ferment lactose because it does not enter the cell easily. Quickly distinguishing these **late lactose fermenters** from true non-fermenters is made possible by supplying them with a lactose structural analog: **o-nitrophenyl-β-D-galactopyranoside (ONPG)**.

When ONPG is made available to bacteria, it freely enters the cells without the aid of a permease. Because of its similarity to β-lactose, it then can become the substrate for any β-galactosidase present. In the reaction that occurs, ONPG is hydrolyzed to β-galactose and o-nitrophenol (ONP), which is yellow (Figs. 5.16 and 5.17, and Table 5-5).

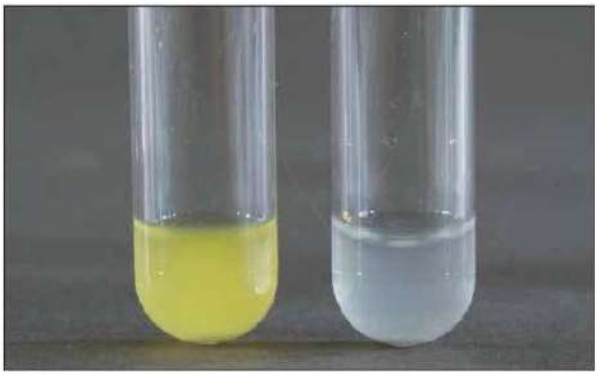
It should be noted that β-galactosidase is an inducible enzyme; it is produced in response to the presence of an appropriate substrate. Therefore, organisms to be tested with ONPG are typically prepared by growing them overnight in a lactose-rich medium, such as Kligler iron agar or triple sugar iron agar, to ensure that they will be actively producing β-galactosidase, given that capability.



5.15 Hydrolysis of Lactose By β-Galactosidase ♦ β-galactosidase is an inducible enzyme in many bacteria; that is, it is produced only in the presence of the inducer lactose. Its activity is to hydrolyze the disaccharide lactose into its component monosaccharides, which can then enter glycolysis.



5.16 Conversion of ONPG to β-Galactose and o-Nitrophenol by β-Galactosidase ♦ ONPG is structurally similar enough to lactose that β-galactosidase will catalyze its hydrolysis into β-galactose and o-nitrophenol. The latter compound is yellow and is indicative of a positive ONPG test.



5.17 ONPG Test Results ♦ ONPG positive (+) is on the left, and ONPG-negative (–) is on the right.

Application

The ONPG test is used to differentiate late lactose fermenters from lactose non-fermenters in the family *Enterobacteriaceae*.

5

In This Exercise

You will be performing the ONPG test using Key Scientific Products' K490 ONPG WEE-TABS. If your instructor uses a different version of the test, he/she will give you instructions to follow based on the manufacturer's recommendations. The organisms chosen for this exercise are two of the enterics that this test was designed to differentiate.

✓ Materials

Per Student Group

- □ Key Scientific Products' K1490 ONPG WEE–TABS
- □ Sterile distilled or deionized water
- □ Sterile transfer pipettes
- □ One sterile wooden stick or disposable inoculating loop (if using *P. vulgaris*)
- □ Fresh Kligler iron agar or triple sugar iron agar cultures of:
  - *Escherichia coli*
  - *Proteus vulgaris* (BSL-2)
  - (Optional) *Morganella morganii*[1]
  - (Optional) *Providencia stuartii*[1]

[1] We recommend testing your strains of *Morganella morganii* and/or *Providencia stuartii*. If either one is ONPG negative, which they generally are (only ~10% of their strains are ONPG positive), we recommend using it in place of *P. vulgaris*.

TABLE 5-5 ONPG Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Yellow color formation	Organism produces β-galactosidase	+
No color change	Organism does not produce β-galactosidase	–

Procedure

1. Dispense 0.5 mL volumes of sterile water into three tubes, and allow the tablets to dissolve.
2. Transfer a heavy inoculum of the test organisms to two tubes. If you are using *P. vulgaris*, perform the transfer with a sterile wooden stick or disposable inoculating loop, and **be sure to employ BSL-2 precautions throughout the exercise.** Do not inoculate the third tube.
3. Incubate all tubes at 35 ± 2°C for 2 hours.
4. Remove all tubes from the incubator and examine them for color changes. Record your results in the table on the data sheet, page 319.

References

DIFCO Laboratories. Page 294 in *DIFCO Manual*, 10th ed. Detroit: DIFCO Laboratories, 1984.

Key Scientific Products' K1490 ONPG WEE–TABs Package Insert. Key Scientific Products. Round Rock, TX.

MacFaddin, Jean F. Page 160 in *Biochemical Tests for Identification of Medical Bacteria*, 2nd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Tille, Patricia M. Page 221 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckenerger, and Gail Woods. Pages 1451–1452 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Philadelphia: Lippincott Williams & Wilkins, 2005.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-5

ONPG Test

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-5, page 318, as a guide, enter your results and interpretations in the table below.

Organism	Result	+/−	Interpretation
Control			

QUESTIONS

1 *When running this test on an unknown organism, why should you run a simultaneous test on a known ONPG-positive organism?*

2 *β-galactosidase is an inducible enzyme.*

a. *What does "inducible" mean?*

b. *What is the purpose of inoculating the ONPG tubes with growth from triple sugar iron agar cultures?*

c. *What would be a possible consequence of inoculating the ONPG medium with an ONPG-positive organism grown on nutrient agar instead of Kligler iron agar or triple sugar iron agar?*

3 *Examine the phenotypes of the organisms listed below. For each organism, predict the ONPG result.*

Organism	β-galactoside permease	β-galactosidase	Predicted ONPG Result (+ or −)
A	Present	Present	
B	Present	Absent	
C	Absent	Present	
D	Absent	Absent	

Catalase Test

EXERCISE 5-6

Theory

The electron transport chains (ETC) of aerobic and facultatively anaerobic bacteria are composed of molecules capable of accepting and donating electrons as conditions dictate. As such, these molecules alternate between the oxidized and reduced forms, passing electrons down the chain to the final electron acceptor, O_2. Energy lost by electrons in this sequential transfer is used to perform oxidative phosphorylation (i.e., produce ATP from ADP).

In most cases, electrons in the aerobic ETC follow the stepwise path to oxygen, but other paths can be followed and these result in production of toxic forms of reduced oxygen[1]. For instance, one ETC carrier molecule called **flavoprotein** can bypass the next carrier in the chain and transfer electrons directly to oxygen (Fig. 5.18), which produces hydrogen peroxide (H_2O_2), a highly potent cellular toxin. $FADH_2$ is capable of the same reaction. Even if the electrons follow the complete ETC another toxin, the superoxide radical (O_2^-), can be produced in the final step because electrons reduce oxygen one at a time and sometimes it is released before it is completely reduced to H_2O.

Hydrogen peroxide and the superoxide radical are toxic because they oxidize biochemicals and make them nonfunctional. However, organisms that produce them also produce enzymes capable of breaking them down. **Superoxide dismutase** catalyzes conversion of superoxide radicals (the more lethal of the two compounds) to hydrogen peroxide (Fig. 5.18). **Catalase** converts hydrogen peroxide into water and gaseous oxygen (Fig. 5.19). In large part (though exceptions exist), the ability to synthesize these protective enzymes accounts for an organism's ability to live in the presence of oxygen (Table 5-6).

Bacteria that produce catalase can be detected easily using typical store-grade hydrogen peroxide. When hydrogen peroxide is added to a catalase-positive culture, oxygen gas bubbles form immediately (Figs. 5.20 and 5.21). If no bubbles appear, the organism is catalase-negative. This test can be performed on a microscope slide or by adding hydrogen peroxide directly to the bacterial growth.

[1] As an analogy, a person can walk down stairs one tread at a time and reach the bottom safely. However, the option exists for jumping from the middle tread to the bottom and spraining an ankle. The person arrives at the same place, but damage is done! The potential energy is not released in a slow, controlled way, but in a larger chunk, which has damaging consequences.

$$FPH_2 + O_2 \longrightarrow FP + H_2O_2$$

Reduced Flavoprotein → Oxidized Flavoprotein; Hydrogen Peroxide

$$2H^+ + 2O_2^- \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2$$

Superoxide Radical → Hydrogen Peroxide

5.18 Microbial Production of H_2O_2 ◆ Hydrogen peroxide may be formed through the transfer of electrons from reduced flavoprotein to oxygen or from the action of superoxide dismutase detoxifying the superoxide radical, another destructive form of reduced oxygen. $FADH_2$ also produces hydrogen peroxide in the same way as flavoprotein.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_{2(g)}$$

Hydrogen Peroxide

5.19 Catalase Mediated Conversion of H_2O_2 ◆ Catalase is an enzyme of aerobes, microaerophiles, and facultative anaerobes that converts hydrogen peroxide to water and oxygen gas.

TABLE 5-6 Protective Enzymes in Various Aerotolerance Groups

Table of Results

Aerotolerance Group	Superoxide Dismutase	Catalase
Obligate aerobe	Present	Present
Facultative anaerobe	Present	Present
Microaerophile	Present	Present in small amounts
Aerotolerant anaerobe	Present	Absent (alternative mechanism)
Obligate anaerobe	Absent	Absent

5.20 Catalase Slide Test Results ♦ Visible bubble production indicates a positive result in the catalase slide test. A catalase-negative organism is on the left. A catalase-positive organism is on the right. For purposes of the photograph, no cover is shown, but one should be used to contain spattering from bubbles produced by a positive reaction (Fig. 5.22).

5.21 Catalase Tube Test Results ♦ The catalase test also may be performed on an agar slant. A catalase-positive organism is on the left. A catalase-negative organism is on the right. Only perform this test when you are done with the culture, because adding hydrogen peroxide makes it unusable.

■ Application

This test is used to identify organisms that produce the enzyme catalase. It is used most commonly to differentiate members of the catalase-positive *Micrococcaceae* from the catalase-negative *Streptococcaceae*. Variations on this test also may be used in identification of *Mycobacterium* species.

■ In This Exercise

You will perform the catalase test. The materials provided will enable you to conduct both the slide test and the slant test. The slide test has to be done first because the tube test requires the addition of hydrogen peroxide directly to the slant, after which the culture will be unusable.

✓ Materials

Per Student

- □ Gloves
- □ Chemical eye protection

Per Student Group

- □ Hydrogen peroxide (3% solution)
- □ Transfer pipettes
- □ Microscope slides
- □ Petri dish base or lid
- □ One nutrient agar slant
- □ Fresh nutrient agar cultures of:
  - *Staphylococcus epidermidis*
  - *Lactococcus lactis*

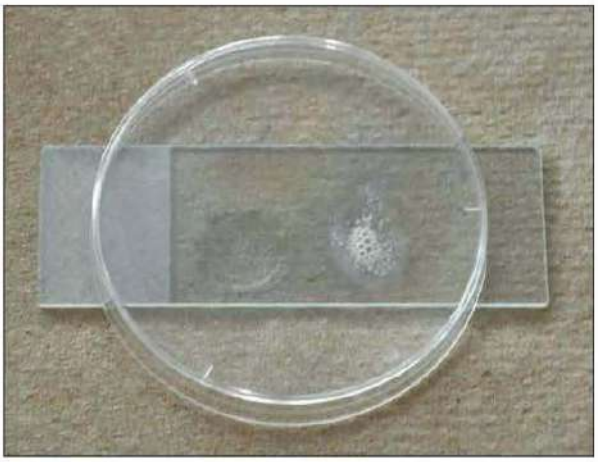
Procedure

Slide Test

1. Transfer a visible amount of growth to a microscope slide with a sterile wooden stick. (If you use a loop, be sure to perform this test in the proper order. Placing the metal loop into H_2O_2 could catalyze a false-positive reaction.) *Note:* It is essential that this test be run with fresh (24-hour) cultures. Older cultures may not have enough active catalase to produce visible bubbles.
2. Aseptically place one or two drops of hydrogen peroxide directly onto the bacteria and immediately cover with the base or lid of a Petri dish (Fig. 5.22). This will contain any spattering due to bubbling of a catalase-positive result. Observe for the formation of bubbles. The cover can be removed when the bubbling has subsided and reused for further testing. Place it on a disinfectant-soaked towel between uses and dispose of it properly when done for the day. *Note:* When running this test on an unknown, a positive control should be run simultaneously to verify the quality of the peroxide.
3. If you do not see obvious bubbling and your instructor permits it, you can observe the slide using the scanning objective on your microscope.
4. Record your results, in the table provided on the data sheet, page 325.

Slant Test

1. Add approximately 1 mL of hydrogen peroxide to each of the three slants (one uninoculated control and two cultures). Do this *one slant at a time*, observing and recording the results as you go. Refer to Table 5-7. This should be done when you are finished with the cultures because they will not be usable for anything else after hydrogen peroxide is added.
2. Record your results in the table provided on the data sheet.



5.22 Cover the Reaction ♦ As soon as you add the hydrogen peroxide to the slide, cover it with a Petri dish lid or base to prevent splattering from a positive result. When the reaction has subsided, the lid can be removed and used again for subsequent tests. Dispose of it properly when finished for the day.

TABLE 5-7 Catalase Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Bubbles	Catalase is present	+
No bubbles	Catalase is absent	–

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 110 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Lányi, B. Page 20 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 78 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Baltimore: Lippincott Williams & Wilkins, 2000.

Smibert, Robert M. and Noel R. Krieg. Page 614 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Stanier, Roger Y., John L. Ingraham, Mark K. Wheelis, and Page R. Painter. Pages 210–211 in *The Microbial World*, 5th ed. Englewood Cliffs, NJ: Prentice-Hall, 1986.

Tille, Patricia M. Pages 97 and 201 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

White, David. Pages 363–364 in *The Physiology and Biochemistry of Prokaryotes*, 2nd ed. New York: Oxford University Press, 2000.

Willey, Joanne M., Linda M. Sherwood, and Christopher J. Woolverton. Pages 144–145 in *Prescott's Principles of Microbiology*. Boston: McGraw-Hill Higher Education, 2009.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-6

Catalase Test

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-7, page 323, as a guide, record your results and interpretations in the table below.

Organism	Bubbles? Y/N	+/−	Interpretation
Uninoculated control			

QUESTIONS

1 *Think about the advice given in the procedure to test a known catalase-positive organism along with an unknown.*

a. *Is this a positive or a negative control?*

b. *What information is provided by the results?*

2 *Consider the step in the tube test where hydrogen peroxide is added to the uninoculated tube.*

a. *Is this a positive or a negative control?*

b. *What information is provided by the results?*

5

3 *When flavoprotein transfers electrons directly to oxygen, hydrogen peroxide is produced. What other consequences might result from electron carriers in the ETC being bypassed?*

4 *Would a false positive from the reaction between the inoculating loop and hydrogen peroxide be caused by poor specificity or poor sensitivity of the test system? Explain.*

EXERCISE
5-7

Oxidase Test

Theory

In the introduction to this section, we discussed the major metabolic reactions and talked a little bit about coenzymes. In Exercise 5-1 Reduction Potential we talked about energy transformations. This test, the oxidase test, is one requiring an understanding of those reactions.

Consider the life of a glucose molecule entering the cell. If it is destined to be used for ATP production, it will become oxidized to CO_2 with the concurrent reduction of the coenzymes NAD^+ and FAD. It is first split into two pyruvate molecules in glycolysis (Fig. A.1) and two NAD^+ are reduced to NADH ($+H^+$). Two ATPs are also made. Then each of the pyruvate molecules becomes oxidized (Fig. A.4), which reduces another NAD^+ to NADH. Concurrently, pyruvate is converted to a two-carbon molecule, which is added to coenzyme A to form acetyl–CoA and one molecule of CO_2. Then the citric acid cycle (Fig. A.4) finishes the oxidation by producing two more molecules of CO_2 (per acetyl–CoA) and reducing three more NAD^+ molecules and one FAD to $FADH_2$. In addition, one ATP is formed per acetyl-Co-A.

As you can see, by this time the cell is becoming quite full of reduced coenzymes. Therefore, in order to continue oxidizing glucose, these coenzymes must be converted back to their oxidized state. This is the job of the electron transport chain (Fig. 5.23).

Many aerobes, microaerophiles, facultative anaerobes, and even some anaerobes have ETCs. The functions of the ETC are to (1) transport electrons down a chain of molecules with increasingly positive reduction potentials (Exercise 5-1) to the terminal electron acceptor ($\frac{1}{2}O_2$, NO_3^-, SO_4^{2-}) and (2) generate a proton motive force by pumping H^+ out of the cell thus creating an ionic imbalance that will drive the production of ATP by way of membrane ATPases. The protons pumped out of the cell come from the hydrogen atoms whose electrons are being transferred down the chain. Because only alternating ETC molecules are able to carry associated protons along with their electrons, the positively charged ions are expelled from the cell. **Flavoproteins, iron-sulfur proteins,** and **cytochromes** are important ETC molecules unable to donate protons.

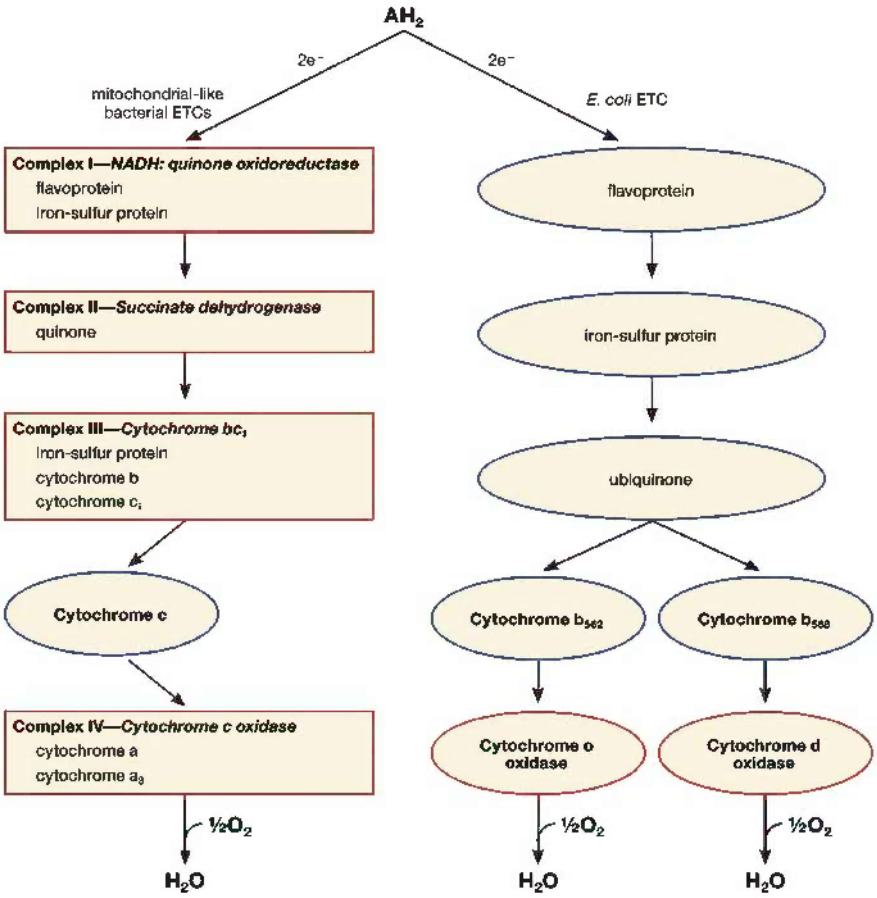
There are many different types of electron transport chains, but all share the characteristics listed above. Some organisms use more than one type of ETC, depending on the availability of oxygen or other terminal electron acceptor. *Escherichia coli*, for example, has two pathways for respiring aerobically and at least one for respiring anaerobically. Many bacteria have ETCs resembling mitochondrial ETCs in eukaryotes. These chains contain a series of four large enzymes broadly named Complexes I, II, III, and IV, each of which contains several molecules jointly able to transfer electrons and use the free energy released in the reactions. The last enzyme in the chain, Complex IV, is called cytochrome c oxidase because it makes the final electron transfer of the chain from cytochrome c, residing in the periplasm or attached to the periplasmic side of the cytoplasmic membrane of Gram-negative cells, to oxygen inside the cell. In Gram-positive cells, it is membrane-bound.

The oxidase test is designed to identify the presence of cytochrome c oxidase. It is able to do this because cytochrome c oxidase has the unique ability to not only oxidize cytochrome c, but to catalyze the *reduction* of cytochrome c by a **chromogenic reducing agent** called tetramethyl-*p*-phenylenediamine. Chromogenic reducing agents are chemicals that develop color as they become oxidized (Fig. 5.24).

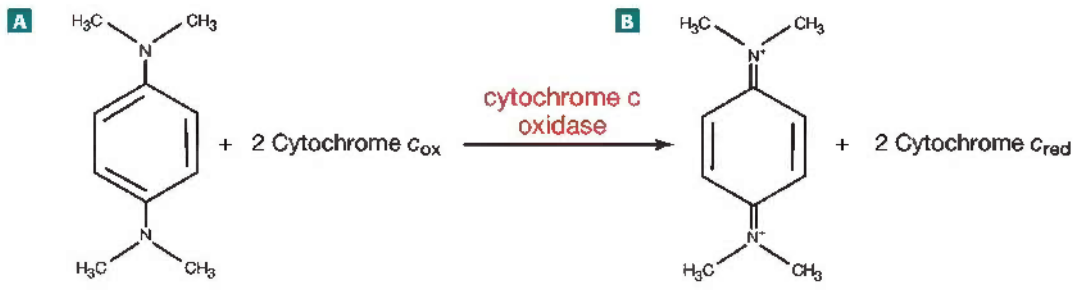
In the oxidase test, the reducing reagent is added directly to bacterial growth on solid media (Fig. 5.25A), or more conveniently, a bacterial colony is transferred to paper and reagent is added (Fig. 5.25B), or even *more* conveniently, bacteria are added to paper already containing the reagent (5.25C). A color change occurs within seconds if the reducing agent becomes oxidized, thus indicating that cytochrome c oxidase is present. Lack of color change within the allotted time means that cytochrome c oxidase is not present and signifies a negative result (Table 5-8).

TABLE 5-8 Oxidase Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Dark blue/purple within 20 seconds	Cytochrome c oxidase is present	+
No color change to blue/purple within 20 seconds	Cytochrome c oxidase not present	–



5.23 Aerobic Electron Transport Chains (ETCs) ♦ Bacterial aerobic electron transport chains (ETCs) show great diversity. However, all begin with flavoproteins that receive electrons from reduced coenzymes, such as NADH or $FADH_2$ (represented by AH_2) and all have proton pumps that create the gradient for ATP synthesis. The part of the ETC relevant to the oxidase, though, is at the end. Bacteria with chains that resemble the mitochondrial ETC in eukaryotes contain cytochrome c oxidase (Complex IV), which transfers electrons from cytochrome c to oxygen. These organisms give a positive result for the oxidase test. Other bacteria, such as members of the *Enterobacteriaceae*, are capable of aerobic respiration, but have a different terminal oxidase system and give a negative result for the oxidase test. Both paths shown on the right are found in *E. coli*. The amount of available O_2 determines which pathway is more active. Enzymatic complexes and oxidases are outlined in red.



5.24 Chemistry of the Oxidase Reaction ♦ (**A**) Cytochrome c oxidase transfers electrons from cytochrome c to O_2, which produces water. (**B**) When in the oxidized state, cytochrome c is available to be reduced by the phenylenediamine reagent. The phenylenediamine reagent is colorless when reduced and purple when oxidized, its condition in a positive oxidase test.

Application

This test is used to identify bacteria containing the respiratory enzyme cytochrome c oxidase. Among its many uses is the presumptive identification of the oxidase-positive *Neisseria* and *Moraxella*. It also can be useful in differentiating the oxidase-negative *Enterobacteriaceae* from the oxidase-positive *Pseudomonadaceae*.

In This Exercise

You will be performing the oxidase test either using one of several commercially available systems or the less expensive versions using "house" reagents (which we describe in the procedure—follow the instructions of the commercial test system if you use one). Your instructor will have chosen the system that is best for your laboratory. Regardless, most oxidase test systems produce a color change from colorless to blue/purple. Reagents used for this test are unstable and may oxidize independently shortly after they become moist. Therefore, it is important to take your readings within the recommended time (usually 20 seconds).

✓ Materials

Per Student

- □ Gloves
- □ Chemical eye protection

Per Student Group

- □ Sterile wooden stick or disposable plastic loop
- □ Small pieces of filter paper (Fig. 5.25B)
- □ Sterile water
- □ Oxidase test reagent (Appendix H)
- □ Fresh slant cultures of:
  - *Escherichia coli*
  - *Neisseria* spp. (e.g., *N. sicca* or *N. flava*)

Procedure

Note: We describe the oxidase test using filter paper and reagent. If you are using a commercial kit, follow its instructions.

1. Place the filter paper on a paper towel. Using a sterile wooden stick or disposable loop, transfer some of the culture to the filter paper. Properly dispose of the stick/loop. ***Note:*** It is essential that this test be run with fresh (24-hour) cultures. Older cultures may not have enough active oxidase to produce a visible color change.
2. Add a few drops of oxidase reagent over the growth and observe for a color change within 20 seconds. ***Note:*** If running an unknown, be sure to also test a known oxidase-positive organism.
3. Repeat steps one and two for the second organism. The same filter paper can be used if there is room. Properly dispose of the filter paper when finished.
4. Enter your results in the table provided on the data sheet, page 331.

5.25 Variations of the Oxidase Test ◆ Many variations of the oxidase test exist, but in all cases reaction with the phenylenediamine reagent produces a blue or purple color. Reading must be taken within 20 seconds because the reagent can react with air and produce a false positive result. **(A)** Shown are oxidase-negative (left) and oxidase-positive (right) bacteria on an agar plate after addition of the phenylenediamine reagent. The organism on the left is its natural color; yellow is not a negative result. **(B)** Alternatively, a small amount of growth can be transferred to a piece of filter paper and reagent added. Shown is a positive result. **(C)** A commercial test system with reagent already in the slide is shown. All the microbiologist has to do is add the organism to a panel and wait 20 seconds for a color change. A positive result is shown in the upper left and a negative result is shown in the upper right panel. (BBL™ DrySlide™ systems available from Becton Dickinson, Sparks, MD.)

References

BBL™ *Dry*Slide™ package insert.

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 116 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Lányi, B. Page 18 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 368 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Pettigrew, Graham W. and Geoffrey R. Moore. Chap. 1 in *Cytochromes c, Biological Aspects*. Springer Series in Molecular Biology. Alexander Rich, ed. Berlin: Springer-Verlag, 1987.

Smibert, Robert M. and Noel R. Krieg. Page 625 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Tille, Patricia M. Pages 97 and 223 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

White, David, Chap. 4 in *The Physiology and Biochemistry of Prokaryotes*, 2nd ed. New York: Oxford University Press, 2000.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-7

Oxidase Test

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-8, page 327, as a guide, enter your results in the table below.

Organism	Color Result	+/−	Interpretation

QUESTIONS

1 *Think about the advice given in the procedure to test a known oxidase-positive organism along with an unknown.*

a. *Is this a positive or a negative control?*

b. *What information is provided by the results?*

2 *Think about the 20-second time limit on the oxidase test.*

a. *What happens to the oxidase reagent after 20 seconds?*

b. *Does this only happen after 20 seconds? If not, why is a 20-second time limit set?*

3 *Provide a possible explanation as to why this test identifies the presence of cytochrome c oxidase and not other oxidases (such as those of* E. coli *shown in Fig. 5.24).*

EXERCISE
5-8
Nitrate Reduction Test

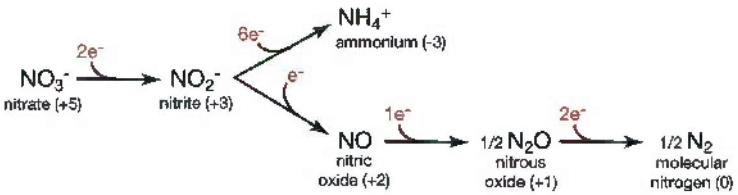
Theory

Anaerobic respiration involves the reduction of (transfer of electrons to) an inorganic molecule other than oxygen. Nitrate reduction is one such example. Many Gram-negative bacteria (including most *Enterobacteriaceae*) contain the enzyme **nitrate reductase** and perform a single-step reduction of nitrate to nitrite ($NO_3^- \rightarrow NO_2^-$). Other bacteria, in a multistep process known as **denitrification**, are capable of enzymatically converting nitrate to molecular nitrogen (N_2). Some products of nitrate reduction are shown in Figure 5.26.

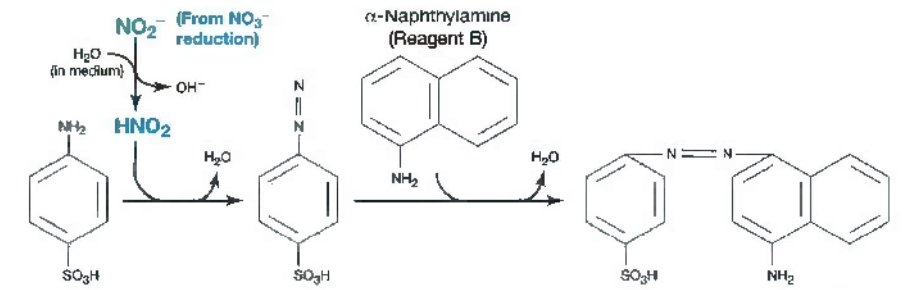
Nitrate broth is an undefined medium of beef extract, peptone, and potassium nitrate (KNO_3). An inverted Durham tube is placed in each broth to trap a portion of any gas produced. In contrast to many differential media, no color indicators are included. The color reactions obtained in nitrate broth take place as a result of reactions between metabolic products and reagents added after incubation (Fig. 5.27).

Before a broth can be tested for nitrate reductase activity (nitrate reduction to nitrite), it must be examined for evidence of denitrification. This is simply a visual inspection for the presence of gas in the Durham tube (Fig. 5.28). If the Durham tube contains gas the test is complete. Denitrification has taken place. Gas produced in a nitrate reduction test by an organism capable of fermenting to gas end products is not determinative because the source of the gas is unknown. For more information on fermentation, see Exercises 5-2 and 5-3.

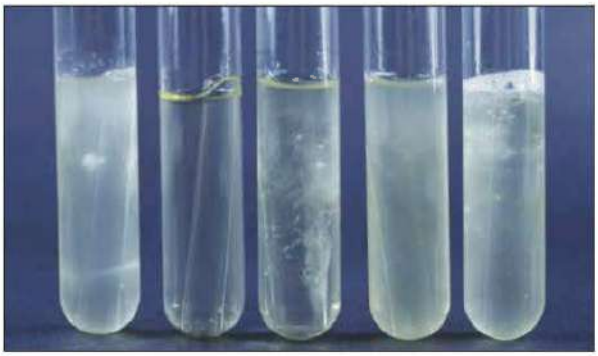
If there is no visual evidence of denitrification (or the test was not determinative), sulfanilic acid (nitrate reagent



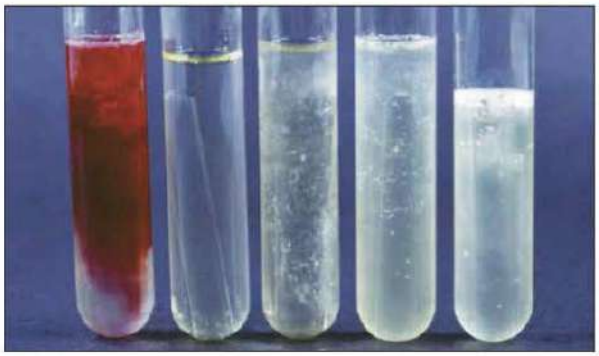
5.26 Possible End Products of Nitrate Reduction ◆ Nitrate reduction is complex. Many different organisms under many different circumstances perform nitrate reduction with many different outcomes. Members of the Enterobacteriaceae simply reduce NO_3 to NO_2. Other bacteria, functionally known as "denitrifiers," reduce NO_3 all the way to N_2 via the intermediates shown, and are important ecologically in the nitrogen cycle. Both of these are anaerobic respiration pathways (also known as "nitrate respiration"). Other organisms are capable of assimilatory nitrate reduction, in which NO_3 is reduced to NH_4^+, which can be used in amino acid synthesis. The oxidation state of nitrogen in each compound is shown in parentheses.



5.27 Nitrate Reduction to Nitrite Indicator Reaction ◆ If nitrate is reduced to nitrite, nitrous acid (HNO_2) will form in the medium. Nitrous acid then reacts with sulfanilic acid (reagent A) to form diazotized sulfanilic acid, which reacts with the α-naphthylamine (reagent B) to form *p*-sulfobenzene-azo-α-naphthylamine, which is red. Thus, a red color indicates the presence of nitrite and is considered a positive result for nitrate reduction to nitrite.



5.28 Incubated Nitrate Broth before the Addition of Reagents ♦ Numbered 1 through 5 from left to right, these are nitrate broths immediately after incubation before the addition of reagents. Tube 2 is an uninoculated control used for color comparison. Note the gas produced by the organism in tube 5. (The bubble in the Durham tube is barely visible behind the layer of bubbles on the surface.) It is a known nonfermenter and, therefore, will receive no reagents. The gas produced is an indication of denitrification and a positive result for nitrate reduction. Tubes 1 through 4 will receive reagents. See Figure 5.29.



5.29 Incubated Nitrate Broth after Addition of Reagents ♦ After the addition of reagents, tube 1 shows a positive result. Tube 3 and tube 4 are inconclusive because they show no color change. Zinc dust must be added to tubes 2 (control), 3, and 4 to verify the presence or absence of nitrate. See Figure 5.30.

5.30 Incubated Nitrate Broth after Addition of Reagents and Zinc ♦ Finally, a pinch of zinc was added to tubes 2, 3, and 4 because they have been colorless up to this point. Tube 2 (the control tube) and tube 3 turned red. This is a negative result because it indicates that nitrate is still present in the tube. Tube 4 did not change color, which indicates that the nitrate was reduced by the organism beyond nitrite to some other nitrogenous compound. This is a positive result.

5

A) and α–naphthylamine (nitrate reagent B) are added to the medium to test for nitrate reduction to nitrite (Fig. 5.29). If present, nitrite will form nitrous acid (HNO_2) in the aqueous medium. Nitrous acid reacts with the added reagents to produce a red, water-soluble compound. Therefore, formation of red color after the addition of reagents indicates that the organism reduced nitrate to nitrite. If no color change takes place with the addition of reagents, the nitrate either was not reduced or was reduced to one of the other nitrogenous compounds shown in Figure 5.26. Because it is visually impossible to tell the difference between these two occurrences, another test must be performed.

In this stage of the test, a small amount of powdered zinc is added to the broth to catalyze the reduction of any nitrate (which still may be present as KNO_3) to nitrite. If nitrate is present at the time zinc is added, it will be converted quickly to nitrite, and the above-described reaction between nitrous acid and reagents will follow and turn the medium red. In this instance, the red color indicates that nitrate was *not* reduced by the organism (Fig. 5.30). No color change after the addition of zinc indicates that the organism reduced the nitrate to NH_4^+, NO, N_2O, or some other nongaseous nitrogenous compound.

Application

Virtually all members of *Enterobacteriaceae* perform a one-step reduction of nitrate to nitrite. The nitrate test differentiates them from Gram-negative rods that either do not reduce nitrate or reduce it beyond nitrite to N_2 or other compounds.

In This Exercise

After inoculation and incubation of the nitrate broths, you first will inspect the Durham tubes included in the media for bubbles—an indication of gas production (Fig. 5.28). One or more of the organisms selected for this exercise are fermenters, and possible producers of gas other than molecular nitrogen; therefore, you will proceed with adding reagents to all tubes. Record any gas observed in the Durham tubes and see if there is a correlation between that and the color results after the reagents and zinc dust have been added. Follow the procedure carefully, and use Table 5-9 as a guide when interpreting the results.

✓ Materials

Per Student

- ☐ Gloves
- ☐ Chemical eye protection

Per Student Group

- ☐ Four nitrate broths
- ☐ Sterile wooden sticks or disposable loops
- ☐ Nitrate test reagents A and B
- ☐ Zinc powder
- ☐ Fresh cultures of:
  - *Alcaligenes faecalis*
  - *Escherichia coli*
  - *Pseudomonas aeruginosa* (BSL-2)

■ Medium and Reagent Recipes

Nitrate Broth

• Beef extract	3.0 g
• Peptone	5.0 g
• Potassium nitrate	1.0 g
• Distilled or deionized water	1.0 L
pH 6.8–7.2 at 25°C	

Reagent A

• *N,N*-Dimethyl-1-naphthylamine	0.6 g
• 5N acetic acid (30%)	100.0 mL

Reagent B

• Sulfanilic acid	0.8 g
• 5N acetic acid (30%)	100.0 mL

Procedure

Lab One

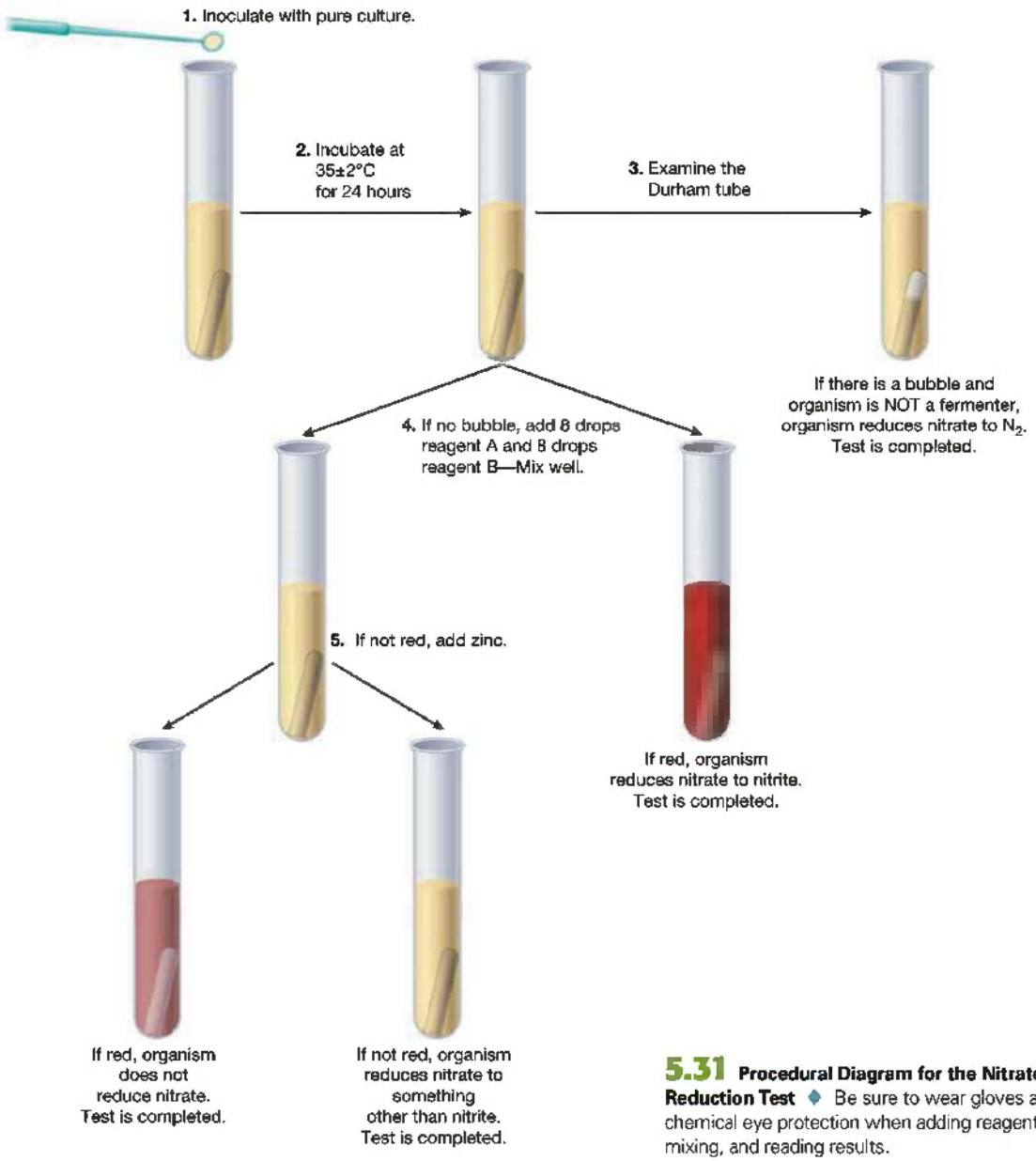
1. Obtain four nitrate broths. Label three of them with the names of the organisms, your name, and the date. Label the fourth tube "control."
2. Inoculate three broths with the test organisms. Do not inoculate the control. Use **BSL-2 precautions with *P. aeruginosa*.**
3. Incubate all tubes at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Wear gloves and chemical eye protection for this part of the procedure.
2. Examine each tube for evidence of gas production. Record your results in the table provided on the data sheet, page 337. Refer to Table 5-9 when making your interpretations. (Be methodical when recording your results. Red color can have opposite interpretations depending on where you are in the procedure.)
3. Using Figure 5.31 as a guide, proceed to the addition of reagents to *all* tubes as follows:
   a. Add eight drops each (approximately 0.5 mL) of reagent A and reagent B to each tube. Mix well, but carefully, and let the tubes stand undisturbed for 10 minutes. Using the control as a comparison, record your test results in the table provided in the data sheet. Set aside any test broths that are positive.
   b. Where appropriate, add a pinch of zinc dust. (Only a small amount is needed. You can dip a wooden applicator into the zinc dust and transfer only the amount that clings to the wood.) Let the tubes stand for 10 minutes. Record your results in the table provided on the data sheet.

TABLE **5-9** **Nitrate Test Results and Interpretations**

Table of Results		
Result	**Interpretation**	**Symbol**
Gas (non-fermenter)	Denitrification—production of nitrogen gas ($NO_3^- \rightarrow NO_2^- \rightarrow N_2$)	+
Gas (fermenter, or status is unknown)	Source of gas is unknown; requires addition of reagents	
Red color (after addition of reagents A and B	Nitrate reduction to nitrite ($NO_3^- \rightarrow NO_2^-$)	+
No color change (after the addition of reagents A and B)	Incomplete test; requires the addition of zinc dust	
No color change (after addition of zinc dust)	Nitrate reduction to nongaseous nitrogenous compounds	+
Red color (after addition of zinc dust)	No nitrate reduction	–


1. Inoculate with pure culture.
2. Incubate at 35±2°C for 24 hours
3. Examine the Durham tube
If there is a bubble and organism is NOT a fermenter, organism reduces nitrate to N_2. Test is completed.
4. If no bubble, add 8 drops reagent A and 8 drops reagent B—Mix well.
5. If not red, add zinc.
If red, organism reduces nitrate to nitrite. Test is completed.
If red, organism does not reduce nitrate. Test is completed.
If not red, organism reduces nitrate to something other than nitrite. Test is completed.

5.31 Procedural Diagram for the Nitrate Reduction Test ♦ Be sure to wear gloves and chemical eye protection when adding reagents, mixing, and reading results.

References

Atlas, Ronald M. and Richard Bartha. Pages 423–425 in *Microbial Ecology—Fundamentals and Applications*, 4th ed. Menlo Park, CA: Benjamin/Cummings Science Publishing, 1998.

Lányi, B. Page 21 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 348 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Moat, Albert G., John W. Foster, and Michael P. Spector. Chap. 14 in *Microbial Physiology*, 4th ed. New York: Wiley-Liss, 2002.

Tille, Patricia M. Page 219 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power, eds. Page 400 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-8

Nitrate Reduction Test

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-9, page 335, as a guide, enter your results and interpretations in the table below.

Organism	Results			Interpretation (Be as specific as possible with respect to end products if positive)
	Gas	Color		
	Y/N	After Reagents	After Zinc	
Uninoculated control				

QUESTIONS

1 *Consider the uninoculated tube.*

a. *Is it a positive or a negative control?*

b. *What information is provided by the uninoculated control?*

2 *Why is gas production not recognized as nitrate reduction when the organism is a known fermenter?*

3 *Suppose you remove your test cultures from the incubator and notice that one of them—a known fermenter—has a gas bubble in the Durham tube. Knowing that fermenters frequently produce gas, you ignore the bubble and proceed to the next step. Adding reagents produces no change, and neither does adding zinc. Is this occurrence consistent with what you have learned about this test? Why?*

4 *Would you change your answer to question 3 if the control broth did not change color after the addition of reagents? What if the control broth had changed color only after the addition of reagents and zinc?*

5 *When testing microaerophiles, some microbiologists prefer to use a semisolid nitrate medium that contains a small amount of agar. Why do you think this is done?*

Nutrient Utilization Tests

In this pair of exercises you will perform tests using two examples of utilization media—Simmons Citrate Medium and Malonate Broth. Utilization media are highly defined formulations designed to differentiate organisms based on their ability to grow when an essential nutrient (e.g., carbon or nitrogen) is strictly limited. For example, citrate medium contains sodium citrate as the only carbon-containing compound and ammonium ion as the only nitrogen source. Malonate broth contains three sources of carbon but prevents utilization of all but one by competitive inhibition of a specific enzyme. ◆

EXERCISE 5-9

Citrate Utilization Test

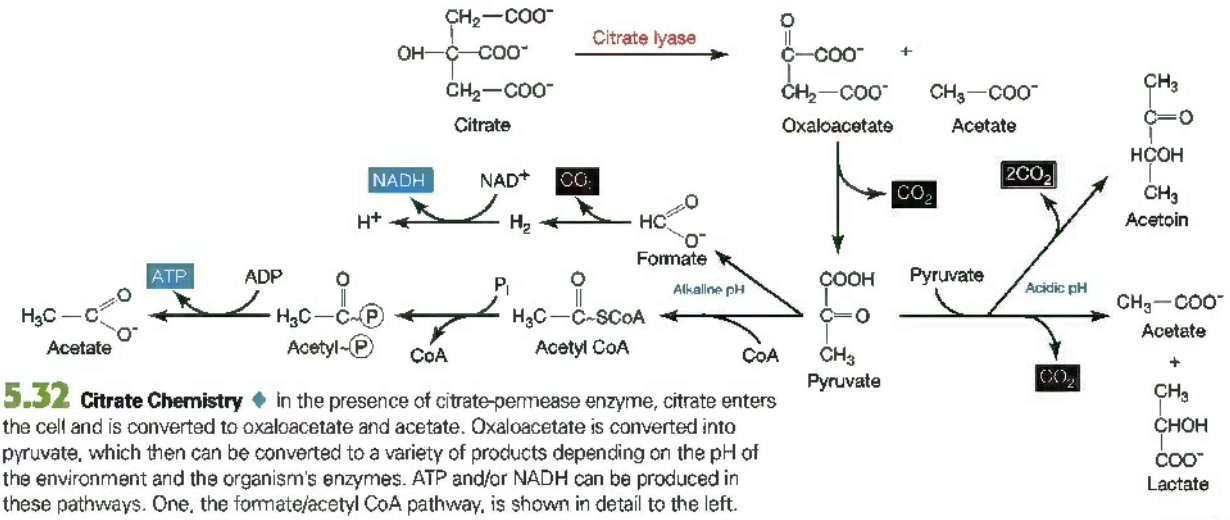
Theory

The citrate utilization test was designed to differentiate members of the *Enterobacteriaceae*, all of which are facultative anaerobes. That is, they have the ability to ferment carbohydrates and they also have the ability to aerobically respire, which means they have a functional citric acid cycle. However, the citrate utilization test does not tell us about the citric acid cycle. Instead, it tells about the ability of organisms to use citrate as their sole carbon source and perform citrate fermentation.

Simmons citrate agar is a defined medium. That is, the amount and source of all ingredients are carefully controlled. Because sodium citrate is the only carbon source in the medium[1] it will not support a complex, high-energy yielding respiratory process like the citric acid cycle. It does, however, provide the means for bacterial species that possess the enzyme **citrate permease** to transport citrate into the cell and perform citrate fermentation. These organisms must also be able to survive with ammonium (in the form of ammonium phosphate) as the sole nitrogen source. Bacteria that do not possess citrate permease will not grow on this medium.

Citrate-positive bacteria hydrolyze citrate into oxaloacetate and acetate using the enzyme **citrate lyase** (Fig. 5.32). From there, oxaloacetate is converted to pyruvate, from which a variety of products can be formed depending on the pH of the environment. Here

[1] Of course, there is CO_2 in the air above the agar, but the test is designed primarily to differentiate *Enterobacteriaceae*, none of which are autotrophic.



5.32 Citrate Chemistry ◆ In the presence of citrate-permease enzyme, citrate enters the cell and is converted to oxaloacetate and acetate. Oxaloacetate is converted into pyruvate, which then can be converted to a variety of products depending on the pH of the environment and the organism's enzymes. ATP and/or NADH can be produced in these pathways. One, the formate/acetyl CoA pathway, is shown in detail to the left.

is the secret: some bacterial fermentation pathways can make ATP and reducing power (NADH or NADPH). For instance, *Klebsiella* and *Enterobacter*, both of which are citrate positive, have the ability to make acetyl-CoA from pyruvate (nothing new) but then have the ability to convert it to acetyl phosphate, which can then be converted to acetate with the production of ATP. In addition, the same bacteria are capable of using H_2 as a source of electrons to reduce NAD and/or NADP. Both of these are shown in Figure 5.32 and are presented as representative examples.

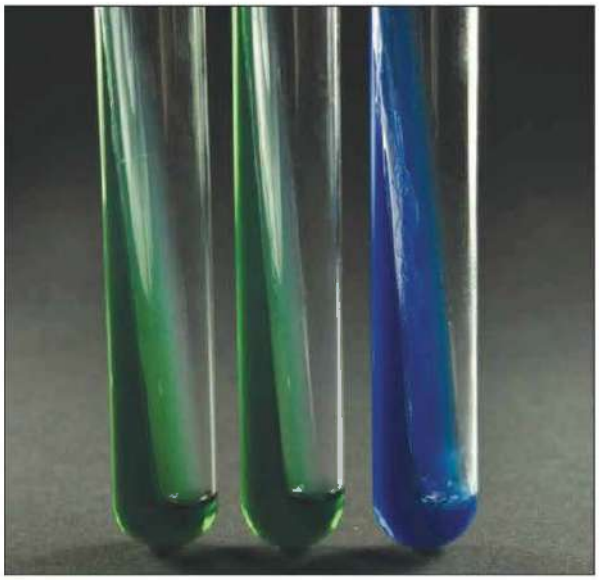
Bacteria that survive in the medium and utilize the citrate also convert the ammonium phosphate to ammonia (NH_3) and ammonium hydroxide (NH_4OH), both of which tend to alkalinize the agar. Bromothymol blue dye, which is green at pH 6.9 and blue at pH 7.6, is an indicator and as the pH goes up, the medium changes from green to blue (Fig. 5.33). Thus, conversion of the medium to blue is a positive citrate test result (Table 5-10). Occasionally a citrate-positive organism will grow on a Simmons citrate slant without producing a change in color. In most cases, this is because of incomplete incubation. In the absence of color change, growth on the slant indicates that citrate is being utilized and is evidence of a positive reaction. To avoid confusion between actual growth and heavy inoculum, which may appear as growth, citrate slants typically are inoculated lightly with an inoculating needle rather than a loop.

TABLE **5-10** **Citrate Test Results and Interpretations**

Table of Results		
Result	**Interpretation**	**Symbol**
Blue (even a small amount)	Citrate is utilized	+
No color change; growth	Citrate is utilized	+
No color change; no growth	Citrate is not utilized	−

Application

The citrate utilization test is used to determine the ability of an organism to use citrate as its sole source of carbon. Citrate utilization is one part of a test series referred to as the IMViC (*I*ndole, *M*ethyl Red, *V*oges-Proskauer and *C*itrate tests) that distinguishes between members of the family *Enterobacteriaceae* and also from other Gram-negative rods.



5.33 **Citrate Test Results** ♦ These Simmons citrate slants were inoculated with a citrate-negative (−) organism on the left and a citrate-positive (+) organism on the right. The slant in the center is an uninoculated control.

In This Exercise

You will be inoculating Simmons citrate slants with a citrate-positive and a citrate-negative organism. To avoid confusing growth on the slant with a heavy inoculum, be sure to inoculate using a needle instead of a loop.

✓ Materials

Per Student Group

- ☐ Three Simmons citrate slants
- ☐ Fresh slant cultures of:
  - *Enterobacter aerogenes*
  - *Escherichia coli*

Medium Recipe

Simmons Citrate Agar

• Ammonium dihydrogen phosphate	1.0 g
• Dipotassium phosphate	1.0 g
• Sodium chloride	5.0 g
• Sodium citrate	2.0 g
• Magnesium sulfate	0.2 g
• Agar	15.0 g
• Bromothymol blue	0.08 g
• Distilled or deionized water	1.0 L

pH 6.7–7.1 at 25°C

Procedure

Lab One

1. Obtain three Simmons citrate tubes. Label two with the names of the organisms, your name, and the date. Label the third tube "control."
2. Using an inoculating needle and *light inoculum*, streak the slants with the test organisms. Do not inoculate the control.
3. Incubate all tubes at 35 ± 2°C for 48 hours.

Lab Two

1. Observe the tubes for color changes and/or growth.
2. Record your results in the table provided on the data sheet, page 343.

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 111 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

MacFaddin, Jean F. Page 98 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Smibert, Robert M. and Noel R. Krieg. Page 614 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Steuber, Julia, Walter Krebs, Michael Bott, and Peter Dimroth. "A Membrane-Bound NAD(P)$^+$-Reducing Hydrogenase Provides Reduced Pyridine Nucleotides during Citrate Fermentation by *Klebsiella pneumoniae*." *J.Bacteriol.* **181**, no. 1 (January 1999): 241–245.

Tille, Patricia M. Page 202 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power, eds. Page 514 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-9

Citrate Utilization Test

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-10, page 340, as a guide, enter your results in the table below.

Organism	Color Result	(+ or −)	Interpretation
Control			

QUESTIONS

1 *Consider the uninoculated tube.*

a. *Is it a positive or a negative control?*

_____

_____

b. *What information is provided by the uninoculated control?*

_____

_____

2 *Many bacteria that are able to metabolize citrate (as seen in the citric acid cycle) produce negative results in this test. Why? Be specific.*

5

3 *Explain how an organism that possesses the citrate lyase enzyme might not test positively on Simmons citrate agar. Is this a false negative result? Why or why not?*

EXERCISE
5-10

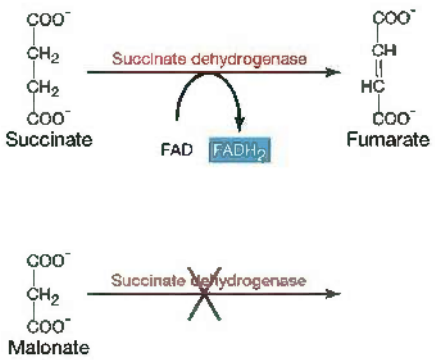
Malonate Utilization Test

Theory

One of the many enzymatic reactions of the citric acid cycle, as illustrated in Appendix A, Figure A.4, is the oxidation of succinate to fumarate. In the reaction, which requires the enzyme **succinate dehydrogenase**, the coenzyme FAD is reduced to $FADH_2$. Refer to the upper reaction in Figure 5.34.

Malonate (malonic acid), which can be added to growth media, is similar enough to succinate to replace it as the substrate in the reaction (Fig. 5.34, lower reaction). This **competitive inhibition** of succinate dehydrogenase, in combination with the subsequent buildup of succinate in the cell, shuts down the citric acid cycle and will kill the organism unless it can ferment or utilize malonate as its sole remaining carbon source.

Malonate broth is the medium used to make this determination. It contains a high concentration of sodium malonate, yeast extract, and a very small amount of glucose to promote growth of organisms that otherwise are slow to respond. Buffers are added to stabilize the medium at pH 6.7. Bromothymol blue dye, which is green in uninoculated media, is added to indicate any shift in pH. If an organism cannot utilize malonate but manages to ferment a small amount of glucose, it may turn the medium slightly yellow or produce no color change at all. These are negative results. If the organism utilizes malonate, it will alkalinize the medium and change the indicator from green to deep blue (Fig. 5.35). Deep blue is positive (Table 5-11).



5.34 Competitive Inhibition of Succinate Dehydrogenase by Malonate ♦ Occupation by malonate of the enzyme succinate dehydrogenase active site prevents the attachment of the normal substrate, succinate and, thus, the conversion of succinate to fumarate in the citric acid cycle.

5.35 Malonate Test Results ♦ Malonate broths illustrating malonate positive (+) on the left, an uninoculated control in the center, and malonate-negative (−) on the right.

5

TABLE 5-11 Malonate Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Dark blue	Malonate is utilized	+
No color change; or slightly yellow	Malonate is not utilized	−

Application

The malonate test was originally designed to differentiate between *Escherichia*, which will not grow in the medium, and *Enterobacter*. Its use as a differential medium has now broadened to include other members of *Enterobacteriaceae*.

In This Exercise

Today, you will inoculate two malonate broths and observe for color changes. Blue color is the only positive for this test; all others are negative.

✓ Materials

Per Student Group

- ☐ Three malonate broths
- ☐ Fresh cultures of:
  - *Enterobacter aerogenes*
  - *Escherichia coli*

Medium Recipe

Malonate Broth

• Yeast extract	1.0 g
• Ammonium sulfate	2.0 g
• Dipotassium phosphate	0.6 g
• Monopotassium phosphate	0.4 g
• Sodium chloride	2.0 g
• Sodium malonate	3.0 g
• Glucose	0.25 g
• Bromothymol blue	0.025 g
• Distilled or deionized water	1.0 L

pH 6.5–6.9 at 25°C

Procedure

5

Lab One

1. Obtain three malonate broths. Label two of them with the names of the organisms, your name, and the date. Label the third tube "control."
2. Inoculate two broths with the test organisms. Do not inoculate the control.
3. Incubate all tubes at 35 ± 2°C for 48 hours.

Lab Two

1. Observe the tubes for color changes.
2. Record your results in the table provided on the data sheet, page 347.

References

MacFaddin, Jean F. Page 310 in *Biochemical Tests for Identification of Medical Bacteria,* 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Zimbro, Mary Jo and David A. Power, eds. Page 343 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media.* Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-10

Malonate Utilization Test

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-11, page 345, as a guide, enter your results in the table below.

Organism	Color Result	(+ or −)	Interpretation
Control			

QUESTIONS

1 *Consider the uninoculated malonate broth.*

a. *Is this a positive or a negative control?*

_____

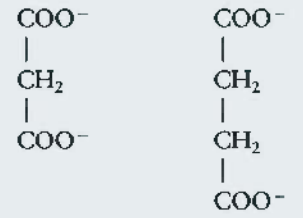
_____

b. *What information is provided by this control?*

_____

_____

2 *Examine the structural formulas below. Ordinarily, in biochemical reactions there is specificity between an enzyme and its substrate. That is, enzymes typically attach to one substrate exclusively. Explain why it is possible for succinate dehydrogenase to bind to two different substrates. Be specific.*



Malonate **Succinate**

3 *The cell has hundreds of succinate dehydrogenase enzymes. How do you suppose malonate concentration affects cell growth?*

4 *How can a yellow color and no color change both be considered negative results for this test?*

Decarboxylation and Deamination Tests

Decarboxylation and deamination tests were designed to differentiate members of *Enterobacteriaceae* and to distinguish them from other Gram-negative rods. Most members of *Enterobacteriaceae* produce one or more enzymes that are necessary to break down amino acids. Enzymes that catalyze the removal of an amino acid's carboxyl group (COOH) are called decarboxylases. Enzymes that catalyze the removal of an amino acid's amine group (NH_2) are called deaminases.

Each decarboxylase and deaminase is specific to a particular substrate, which in these two exercises means a different amino acid. Decarboxylases catalyze reactions that produce alkaline products. Thus, we are able to identify the ability of an organism to produce a specific decarboxylase by preparing base medium, adding a known amino acid, and including a pH indicator to mark the shift to alkalinity. Differentiation of an organism in deamination media employs the same principle of substrate exclusivity by including a single known amino acid but requires the addition of a chemical reagent to produce a readable result.

In the next two exercises you will be introduced to the most common of both types of tests. In Exercise 5-11 you will test bacterial ability to decarboxylate the amino acids arginine, lysine, and ornithine. In Exercise 5-12 you will test bacterial ability to deaminate the amino acid phenylalanine. ◆

EXERCISE 5-11

Decarboxylation Tests

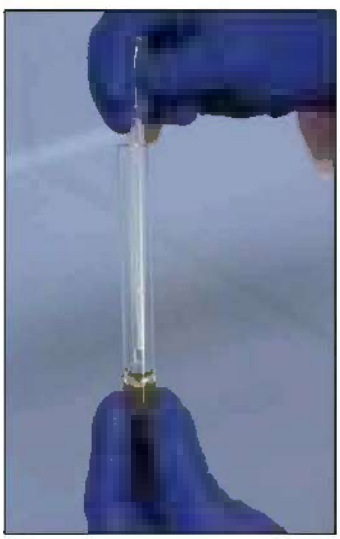
Theory

Møller's decarboxylase base medium contains peptone, glucose, the pH indicator bromcresol purple, and the coenzyme pyridoxal phosphate. Bromcresol purple is purple at pH 6.8 and above, and yellow below pH 5.2. Base medium can be used with one of a number of specific amino acid substrates, depending on the decarboxylase to be identified.

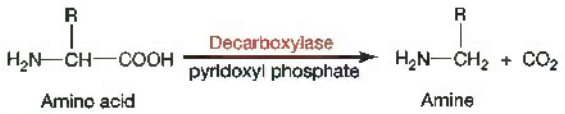
After inoculation, an overlay of mineral oil is used to seal the medium from external oxygen and promote fermentation (Fig. 5.36). Glucose fermentation in the anaerobic medium initially turns it yellow because of the accumulation of acid end products. The low pH and presence of the specific amino acid induces decarboxylase-positive organisms to produce the enzyme. (That is, the specific decarboxylase gene is "switched on.")

Decarboxylation of the amino acid results in accumulation of alkaline end products (Figs. 5.37 through 5.40) that turn the medium purple. If the organism is a glucose fermenter (as are all of the *Enterobacteriaceae*) but does not produce the appropriate decarboxylase, the medium will turn yellow and remain so. If the organism does not ferment glucose, the medium will exhibit no color change. Purple color is the only positive result; all others are negative (Fig. 5.41).

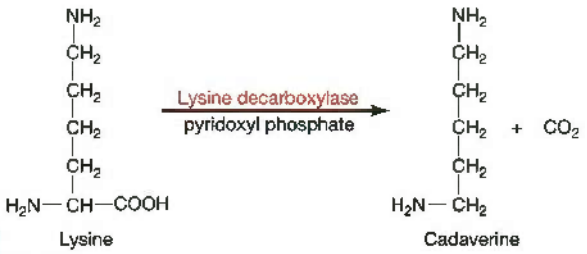
Arginine decarboxylase medium may, in fact, identify a second pathway for arginine catabolism. It is called the arginine dihydrolase system. In it, arginine is converted to citrulline and then into ornithine, a CO_2, and two NH_3, which lowers the pH and turns the medium purple. If the species is also positive for ornithine decarboxylase, it will produce putrescine and CO_2 from ornithine (Fig. 5.39).



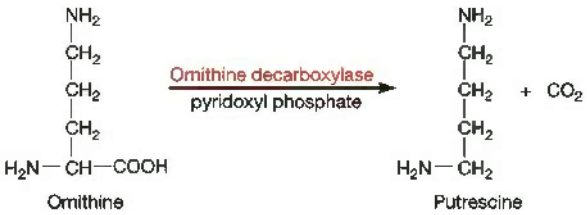
5.36 Adding Sterile Mineral Oil to the Tubes ◆ Gently let the oil slide down the side of the test tube to a depth of approximately 4 mm (not mL). It is important to keep the mineral oil sterile to prevent contamination.



5.37 Amino Acid Decarboxylation ♦ Removal of an amino acid's carboxyl group results in the formation of an amine and carbon dioxide.



5.38 Lysine Decarboxylation ♦ Decarboxylation of the amino acid lysine produces cadaverine and CO_2.



5.39 Ornithine Decarboxylation ♦ Decarboxylation of the amino acid ornithine produces putrescine and CO_2.

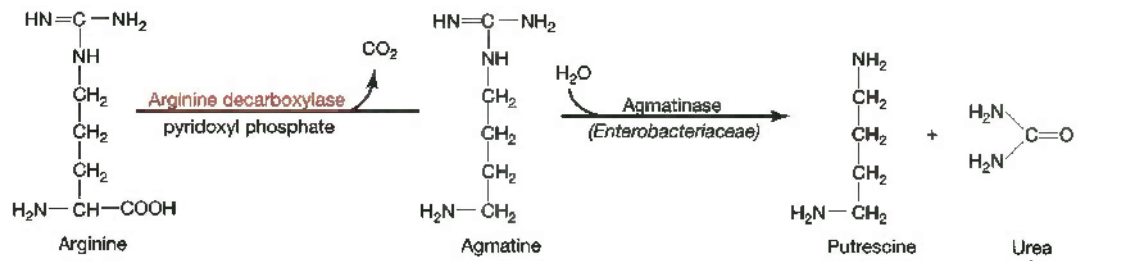
Application

Decarboxylase media can include any one of several amino acids. Typically, these media are used to differentiate organisms in the family *Enterobacteriaceae* and to distinguish them from other Gram-negative rods. The most frequently used are arginine, lysine, and ornithine.

In This Exercise

You will perform decarboxylation tests on three organisms in Møller's arginine, lysine, ornithine, and base decarboxylase media. This procedure requires the aseptic addition of mineral oil. Be careful not to introduce contaminants while adding the oil. Refer to Figure 5.41 and Table 5-12 when interpreting your results.

5.41 Decarboxylation Test Results ♦ A lysine decarboxylase-negative (−) result is on the left, an uninoculated control is in the center, and a lysine decarboxylase-positive (+) is on the right. The color results shown here are representative of arginine and ornithine decarboxylase media as well.



5.40 Arginine Decarboxylation ♦ Decarboxylation of the amino acid arginine produces the amine agmatine. Members of *Enterobacteriaceae* are capable of degrading agmatine into putrescine and urea. Those strains with urease can break down the urea further into ammonia and carbon dioxide. Thus, the end products of arginine catabolism are carbon dioxide, putrescine and urea, *or* (in the presence of urease) carbon dioxide, putrescine, and ammonia. Arginine may also be catabolized by a dihydrolase enzyme, with the production of ornithine, NH_3, and CO_2. The dihydrolase pathway is indistinguishable from the decarboxylase pathway because both produce alkaline end products. In ornithine decarboxylase-positive organisms, the ornithine is further degraded into putrescine and CO_2.

TABLE **5-12** Decarboxylase Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
No color change	No decarboxylation	–
Yellow	Fermentation; no decarboxylation	–
Purple (may be slight)	Decarboxylation; organism produces the specific decarboxylase enzyme	+

✓ Materials

Per Student Group

- ☐ Four lysine decarboxylase broths
- ☐ Four ornithine decarboxylase broths
- ☐ Four arginine decarboxylase broths
- ☐ Four decarboxylase base media
- ☐ Sterile mineral oil in microtubes
- ☐ Sixteen sterile transfer pipettes
- ☐ Recommended organisms:
  - *Enterobacter aerogenes*
  - *Enterobacter cloacae*
  - *Providencia stuartii*

■ Medium Recipe

Decarboxylase Medium (Møller)

• Peptone	5.0 g
• Beef extract	5.0 g
• Glucose (dextrose)	0.5 g
• Bromocresol purple	0.01 g
• Cresol red	0.005 g
• Pyridoxal	0.005 g
• L-Lysine, L-Ornithine, or L-Arginine	10.0 g
• Distilled or deionized water	1.0 L

pH 5.8–6.2 at 25°C

Procedure

Lab One

1. Obtain four of each decarboxylase broth. Label three of each with the name of the organism, your name, and the date. Label the fourth of each broth "control."
2. Inoculate the media with the test organisms. Do not inoculate the control.
3. Overlay all tubes with 3 to 4 mm sterile mineral oil (Fig. 5.36).
4. Incubate all tubes aerobically at 35 ± 2°C for 96 hours (up to one week).

Lab Two

1. Remove the tubes from the incubator and examine them for color changes.
2. Record your results in the table provided on the data sheet, page 353.

5

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 111 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

DIFCO Laboratories. Page 268 in *DIFCO Manual*, 10th ed. Detroit: DIFCO Laboratories, 1984.

Forbes, Betty A., Daniel F. Sahm, and Alice S. Weissfeld. Page 267 in *Bailey & Scott's Diagnostic Microbiology*, 11th ed. St. Louis, MO: Mosby, 2002.

Lányi, B. Page 29 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 120 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Tille, Patricia M. Page 204 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-11

Decarboxylation Tests

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-12, page 351, as a guide, enter your results in the table below.

Organism	Results								Interpretation
	Lysine		Ornithine		Arginine		Base		
	Color	+/−	Color	+/−	Color	+/−	Color	+/−	
Control									

QUESTIONS

1 *Consider the decarboxylase base broth.*

a. *Why isn't a different base broth required for each decarboxylase test?*

b. *Is base broth a positive or a negative control?*

c. *What information is provided by the base broth?*

2 *Consider the uninoculated tubes.*

a. *Are the uninoculated tubes positive or negative controls?*

b. *What information do the uninoculated tubes provide?*

c. *Would it be acceptable to use uninoculated ornithine decarboxylase medium as a control for, say, arginine decarboxylase tests? Defend your answer.*

3 *How can no color change in the broth and a conversion to a yellow color both be considered negative results?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-11

(continued)

4 *Incubation time for this medium is 96 hours.*

a. *Under what circumstances would an early reading be allowable? Explain.*

b. *Under what circumstances would an early reading not be allowable? Explain.*

5 *Think about the effect of the mineral oil on the test results.*

a. *Would omitting the sterile mineral oil affect the specificity of the test? Explain.*

b. *Would omitting the sterile mineral oil affect the sensitivity of the test? Explain.*

Phenylalanine Deaminase Test

EXERCISE 5-12

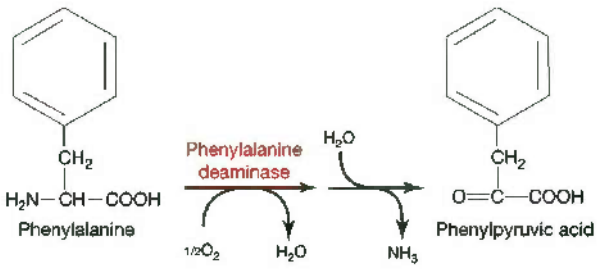
Theory

In Exercise 5-11 we saw one way amino acids can be catabolized—that is, by removal of the carboxyl group. In this lab we will examine a second way, and that is by the removal of the amine group (NH_2) in a process called **deamination** (Fig. 5.42).

Organisms that produce phenylalanine deaminase can be identified by their ability to deaminate the amino acid phenylalanine. The reaction, as shown in Figure 5.43, initially removes two hydrogen ions, which combine with oxygen to make water and produce an intermediate acid. The intermediate is then deaminated to produce ammonia (NH_3) and phenylpyruvic acid. Deaminase activity, therefore, is evidenced by the presence of phenylpyruvic acid.

Phenylalanine agar provides a rich source of phenylalanine. A reagent containing ferric chloride ($FeCl_3$) is added to the medium after incubation. The normally colorless phenylpyruvic acid reacts with the ferric chloride and turns a dark green color almost immediately (Fig. 5.44). Formation of green color indicates the presence of phenylpyruvic acid and, hence, the presence of phenylalanine deaminase. Yellow is negative (Fig. 5.45).

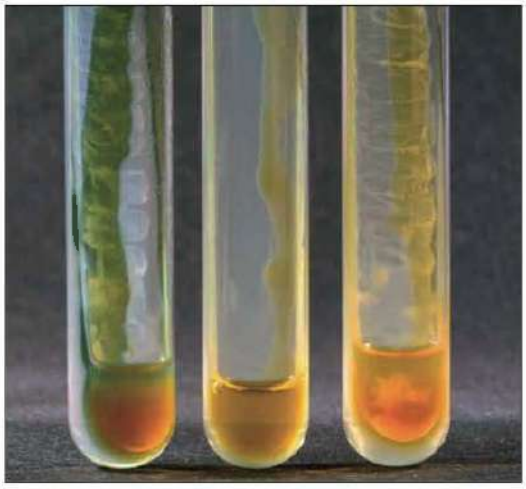
5.42 Amino Acid Deamination ♦ Deamination is the removal of an amine (NH_2) from an amino acid.



5.43 Deamination of Phenylalanine ♦ Phenylalanine deamination occurs as a two-step process, but the net result is phenylpyruvic acid and ammonia.

Phenylpyruvic Acid + $FeCl_3$ ⟶ Green Color

5.44 Phenylalanine Deaminase Test Indicator Reaction ♦ Phenylpyruvic acid produced by phenylalanine deamination reacts with $FeCl_3$ to produce a green color and indicates a positive phenylalanine deaminase test. Be sure to read the result immediately, as the color may fade.



5.45 Phenylalanine Deaminase Test Results ♦ Note the color produced by the stream of ferric chloride in each tube. A phenylalanine deaminase-positive (+) organism is on the left, an uninoculated control is in the middle, and a phenylalanine deaminase-negative (−) organism is on the right.

Application

This medium is used to differentiate the genera *Morganella*, *Proteus*, and *Providencia* from other members of the *Enterobacteriaceae*.

In This Exercise

You will inoculate two phenylalanine agar slants with test organisms. After incubation you will add 12% $FeCl_3$ (phenylalanine deaminase test reagent) and watch for a color change. Refer to Figure 5.45 and Table 5-13 when making your interpretations and recording your results.

TABLE **5-13** Phenylalanine Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Green color	Phenylalanine deaminase is present	+
No color change	Phenylalanine deaminase is absent	–

✓ Materials

Per Student

- □ Gloves
- □ Chemical eye protection

Per Student Group

- □ Three phenylalanine agar slants
- □ 12% ferric chloride solution
- □ Fresh slant cultures of:
  - *Escherichia coli*
  - *Providencia stuartii*

■ Medium and Reagent Recipes

Phenylalanine Agar

• DL-Phenylalanine	2.0 g
• Yeast extract	3.0 g
• Sodium chloride	5.0 g
• Sodium phosphate	1.0 g
• Agar	12.0 g
• Distilled or deionized water	1.0 L
pH 7.1–7.5 at 25°C	

Phenylalanine Deaminase Test Reagent

• Ferric chloride	12.0 g
• Concentrated hydrochloric acid	2.5 mL
• Deionized water (to bring the total to)	100.0 mL

Procedure

Lab One

1. Obtain three phenylalanine agar slants. Label two slants with the name of the organism, your name, and the date. Label the third slant "control."
2. Streak two slants with heavy inocula of the test organisms. Do not inoculate the control.
3. Incubate all slants aerobically at 35 ± 2°C for 18 to 24 hours.

Lab Two

1. Be sure to wear gloves and chemical eye protection as you add the indicator and read the results.
2. Add a few drops of 12% ferric chloride solution to each tube and observe for color change. (*Note*: This color may fade quickly, so read and record your results immediately.)
3. Record your results in the table provided on the data sheet, page 359.

References

Lányi, B. Page 28 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 388 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Tille, Patricia M. Page 225 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power, eds. Page 442 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-12

Phenylalanine Deaminase Test

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-13, page 358, as a guide, enter your results in the table below.

Organism	Color Result	(+ or −)	Interpretation
Control			

QUESTIONS

1 *Consider the uninoculated phenylalanine agar tube.*

a. *Is this a positive or a negative control?*

_____

_____

_____

b. *What information is provided by this control?*

_____

_____

_____

2 *If you are performing this test on an unknown organism, why is it a good idea to run simultaneous tests on known phenylalanine-positive and phenylalanine-negative organisms?*

3 *What do deamination and decarboxylation reactions have in common?*

4 *Phenylalanine medium sometimes is prepared in broth form with a pH indicator similar to that used in decarboxylase medium so that increases in pH then can be detected by development of purple color. (In order to answer these questions you would need to have performed the decarboxylase test.)*

a. *When testing an organism for the ability to deaminate phenylalanine, would you expect to overlay this medium with mineral oil? Why or why not?*

b. *If you ran such a test, how would you know that an increase in pH was due to deamination and not to decarboxylation?*

Tests Detecting Hydrolytic Enzymes

Reactions that use water to split complex molecules are called **hydrolysis** (or **hydrolytic**) reactions. The enzymes required for these reactions are called hydrolytic enzymes. When the enzyme catalyzes its reaction inside the cell, it is referred to as **intracellular**. Enzymes secreted from the organism to catalyze reactions outside the cell are called **extracellular** enzymes, or **exoenzymes**. In this set of exercises you will be performing tests that identify the actions of both intracellular and extracellular types of enzymes.

Starch agar, DNase agar, tributyrin agar, and milk agar are plated media that identify extracellular enzymes capable of diffusing into the medium and producing a distinguishable halo of clearing around the bacterial growth. Nutrient gelatin detects gelatinase—an exoenzyme that liquefies the solid medium. The hydrolytic enzymes detected by the Urease Test are intracellular and produce identifiable color changes in the medium. Lastly, the PYR Test identifies a specific aminopeptidase that removes the amino acid on the amino end of a polypeptide. ◆

Starch Hydrolysis (Amylase Test)

EXERCISE 5-13

5

Theory

Starch is a polysaccharide made up of α-D-glucose subunits. It exists in two forms—linear (amylose) and branched (amylopectin)—usually as a mixture with the branched configuration being predominant. The α-D-glucose molecules in both amylose and amylopectin are bonded by 1,4-α-glycosidic (acetal) linkages (Fig. 5.46). The two forms differ in that amylopectin contains polysaccharide side chains connected to approximately every 30th glucose in the main chain. These side chains are identical to the main chain except that the number 1 carbon of the first glucose in the side chain is bonded to carbon number 6 of the main chain glucose. The bond, therefore, is a 1,6-α-glycosidic linkage.

Starch is too large to pass through the bacterial cell membrane. Therefore, to be of metabolic value to the bacteria, it first must be split into smaller fragments or individual glucose molecules. Organisms that produce and secrete the extracellular enzymes **α-amylase** and **oligo-1,6-glucosidase** are able to hydrolyze starch by breaking the glycosidic linkages between sugar subunits. As shown in Figure 5.46, the end result of these reactions is the complete hydrolysis of the polysaccharide to its individual α-glucose subunits.

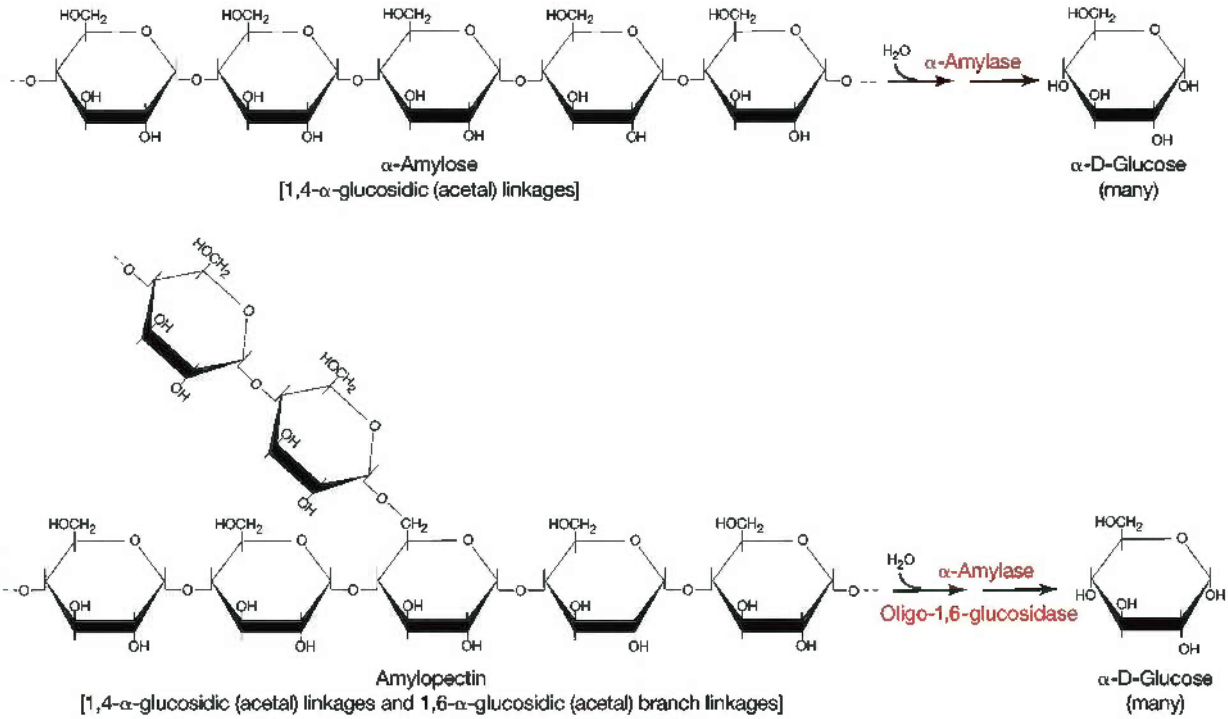
Starch agar is a simple plated medium of beef extract, soluble starch, and agar. When organisms that produce α-amylase and oligo-1,6-glucosidase are cultivated on starch agar, they hydrolyze the starch in the area surrounding their growth. Because both starch and its sugar subunits are virtually invisible in the medium, the reagent iodine is used to detect the presence or absence of starch in the vicinity around the bacterial growth. Iodine reacts with starch and produces a blue or dark brown color; therefore, any microbial starch hydrolysis will be revealed as a clear zone surrounding the growth (Fig. 5.47 and Table 5-14).

Application

Starch agar originally was designed for cultivating *Neisseria*. It no longer is used for this, but with pH indicators, it is used to isolate and presumptively identify *Gardnerella vaginalis*. It aids in differentiating members of the genera *Corynebacterium, Clostridium, Bacillus, Bacteroides, Fusobacterium*, and *Enterococcus*, most of which have (+) and (−) species.

In This Exercise

You will inoculate a starch agar plate with two organisms. After incubation you will add a few drops of iodine to reveal clear areas surrounding the growth. Iodine will not color the agar where there is growth, only the area surrounding the growth. Before you add the iodine, make a line at the margins of growth on the plate so you do not confuse thinning growth at the edges with the halo produced by starch hydrolysis.



5.46 Starch Hydrolysis by α-Amylase and Oligo-1,6-Glucosidase ♦ The enzymes α-amylase and oligo-1,6-glucosidase hydrolyze starch by breaking glycosidic linkages, resulting in release of the component glucose subunits.

5.47 Starch Hydrolysis Test ♦ (A) This is a starch agar plate inoculated with two organisms before iodine has been added. Notice the wavy margins of both organisms. (B) This is the same plate as in photo A after iodine has been added. The clearing in the medium around the organism on the left demonstrates a positive result for starch hydrolysis. The organism on the right, with no clearing, is negative. Note that the wavy margin of the negative organism produced a lighter region on the periphery of the growth that might be misinterpreted as clearing. To prevent recording a false positive, it is a good idea to establish the edges of growth of each organism by drawing a line on the plate's base prior to adding iodine.

TABLE **5-14** Amylase Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Clearing around growth	α-amylase and/or oligo-1,6-glucosidase is present	+
No clearing around growth	Neither α-amylase nor oligo-1,6-glucosidase are present	–

✓ Materials

Per Student Group

- ☐ One starch agar plate
- ☐ Gram iodine (from your Gram stain kit)
- ☐ Recommended organisms:
  - *Bacillus subtilis*
  - *Escherichia coli*

■ Medium Recipe

Starch Agar

• Beef extract	3.0 g
• Soluble starch	10.0 g
• Agar	12.0 g
• Distilled or deionized water	1.0 L
pH 7.3–7.7 at 25°C	

Procedure

Lab One

1. Using a marking pen, divide the starch agar plate into three equal sectors. Be sure to mark on the bottom of the plate, not the lid.
2. Label the plate with the names of the organisms, your name, and the date.
3. Spot inoculate two sectors with the test organisms near the edge of the plate.
4. Invert the plate and incubate it aerobically at 35 ± 2°C for 48 hours.

Lab Two

1. Remove the plate from the incubator, and, before adding the iodine, note the location and appearance of the growth. Use a marking pen to indicate the margins of the growth.
2. Cover the growth and surrounding areas with Gram iodine. Do not flood the plate, but do put enough iodine on to colorize the agar completely. Allow the iodine to soak in. (Use a transfer pipette to remove any excess iodine.) Examine the areas surrounding the growth for clearing. (Usually the growth on the agar prevents contact between the starch and the iodine so no color reaction takes place at that point. Beginning students sometimes look at this lack of color change and judge it incorrectly as a positive result. Therefore, when examining the agar for clearing, look for a halo *around* the growth, not at the growth itself.)
3. Record your results in the table provided on the data sheet, page 365.

5

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 117 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Lányi, B. Page 55 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 412 in *Biochemical Tests for Identification of Medical Bacteria*, 2nd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Smibert, Robert M. and Noel R. Krieg. Page 630 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Zimbro, Mary Jo and David A. Power, eds. *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-13

Starch Hydrolysis (Amylase Test)

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-14, page 363, as a guide, enter your results in the table below.

Organism	**Result**	(+/−)	**Interpretation** (Include Enzyme)
Control sector			

QUESTIONS

1 *Suppose you had poured iodine on your plate and noticed clearings in the uninoculated area, as well as around both of your transferred cultures.*

a. *What are some possible explanations for this occurrence?*

b. *Was integrity of the test compromised?*

c. *What measures could be taken to avoid this problem in the future?*

2 *How would you expect both positive and negative results to be affected if you were to add glucose to the medium?*

5

3 *In many tests it is acceptable to read a positive result before the incubation time is completed. Why is this not the case with starch agar?*

4 *Suppose you could selectively prevent production of α-amylase or oligo-1,6-glucosidase in an organism that normally hydrolyzes starch. Which enzyme would the organism miss the most?*

DNA Hydrolysis (DNase Test)

EXERCISE 5-14

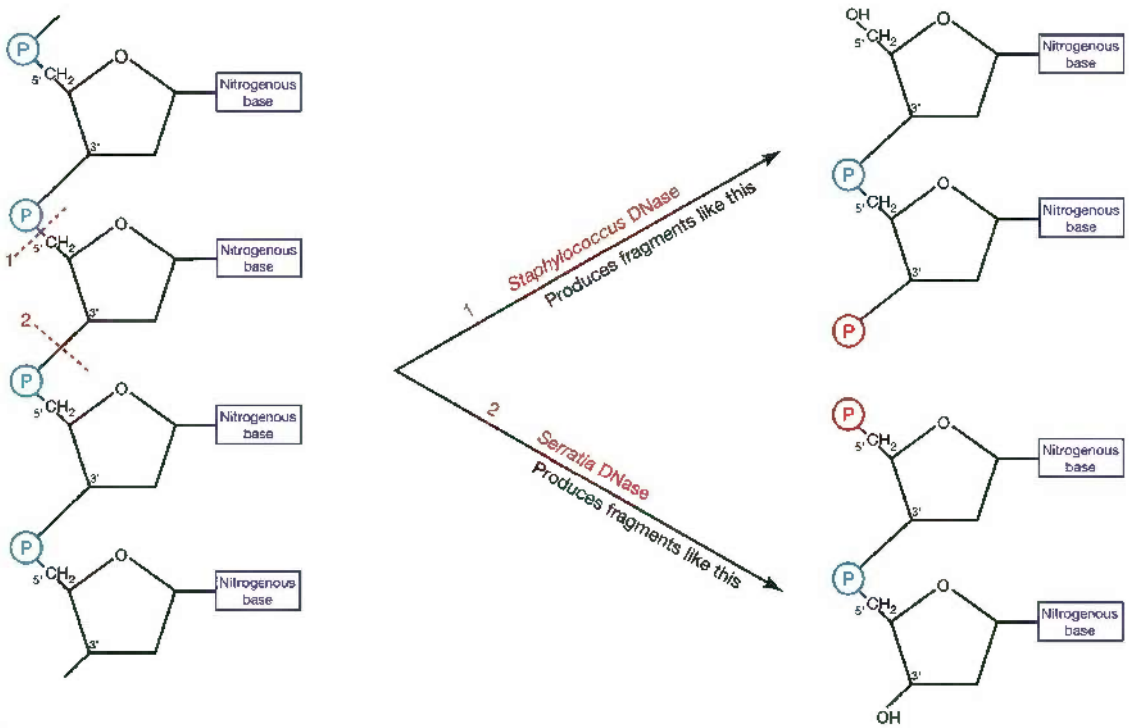
Theory

An enzyme that catalyzes the depolymerization of DNA into small fragments (oligonucleotides) or single nucleotides is called a **deoxyribonuclease**, or **DNase** (Fig. 5.48). DNase is an exoenzyme; that is, an enzyme that is secreted by a cell and acts on the substrate extracellularly. Extracellular digestion typically allows utilization of a macromolecule too large to be transported into the cell. Ability to produce this enzyme can be determined by culturing and observing an organism on a DNase test agar plate.

DNase test agar contains peptides derived from soybean and casein that serve as carbon and nitrogen sources, sodium chloride for osmotic balance, DNA as the substrate, and methyl green dye as an indicator of DNase activity. DNA also serves as an additional carbon and nitrogen source. The dye forms a complex with polymerized DNA and gives the agar a blue-green color, but no complex is formed with nucleotides. Therefore, clearing that develops around bacterial growth on the medium is an indication of DNase activity (DNA hydrolysis) and is considered a positive result (Fig. 5.49).

Application

DNase test agar is used to distinguish *Serratia* species (+) from *Enterobacter* species (–), *Moraxella catarrhalis* (+) from *Neisseria* species (–), and *Staphylococcus aureus* (+) from other *Staphylococcus* species (–).



5.48 Two Patterns of DNA Hydrolysis ◆ DNase from *Staphylococcus* hydrolyzes DNA at the bond between the 5'-carbon and the phosphate (illustrated by line 1), thereby producing fragments with a free 3'-phosphate (shown in red on the upper fragment at the right). Most fragments are one or two nucleotides long; a dinucleotide is shown here. Note that fragments cut by this DNase will not have a 5'-phosphate because it will be on the 3'-carbon of the next fragment. *Serratia* DNase cleaves the bond between the phosphate and the 3'-carbon (illustrated by line 2) and produces fragments with free 5'-phosphates (shown in red on the lower fragment at the right). Most fragments are two to four nucleotides in length; a dinucleotide is shown here. Note that the 3' end on each fragment cut this way would have a hydroxyl group, not a phosphate.

5.49 DNA Hydrolysis Test Results on DNase Agar with Methyl Green ◆ This DNase test agar plate was inoculated with two DNase-positive (+) organisms (top and lower left) and a DNase-negative (−) organism (lower right).

5

In This Exercise

You will inoculate one DNase plate with two of the organisms the test was designed to differentiate. For best results, limit incubation time to 24 hours. Depending on the age of the plates and the length of incubation, the clearing around a DNase-positive organism may be subtle and difficult to see. When reading the plate, it might be helpful to look through it while holding it several inches above a white piece of paper. Use Table 5-15 to assist in interpretation.

TABLE 5-15 DNA Hydrolysis Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Clearing in agar (loss of green color) around growth	DNAse is present	+
No clearing in agar around growth	DNAse is absent	−

✓ Materials

Per Student Group

- ☐ One DNase test agar plate
- ☐ Fresh cultures of:
  - *Serratia marcescens*
  - *Enterobacter aerogenes*

Medium Recipe

DNase Test Agar with Methyl Green

• Tryptose	20.0 g
• Deoxyribonucleic acid	2.0 g
• Sodium chloride	5.0 g
• Agar	15.0 g
• Methyl green	0.05 g
• Distilled or deionized water	1.0 L

pH 7.1–7.4 at 25°C

Procedure

Lab One

1. Using a marking pen, divide the DNase test agar plate into three sectors. Be sure to mark on the bottom of the plate, not the lid.
2. Label the plate with the names of the organisms, your name, and the date.
3. Spot inoculate two sectors with the test organisms and leave the third sector as a control.
4. Invert the plate and incubate it aerobically at 35 ± 2°C for 24 hours. (If your labs are scheduled more than 24 hours apart, arrange to have someone place your plates in a refrigerator until you can examine them.)

Lab Two

1. Remove the plates from the incubator (or refrigerator) and examine the agar for clearing around the bacterial growth.
2. Record your results in the table provided on the data sheet, page 369.

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 114 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Delost, Maria Dannessa. Page 111 in *Introduction to Diagnostic Microbiology*. St. Louis, MO: Mosby, 1997.

Lányi, B. Page 33 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 136 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Tille, Patricia M. Page 205 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power, eds. Page 170 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-14

DNA Hydrolysis (DNase Test)

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-15, page 368, as a guide, record your DNase results in the table below.

Organism	Result	(+/−)	Interpretation (include enzyme)
Control sector			

QUESTIONS

1 *In Theory on page 367 of this exercise, the disassembly of DNA was described as a "depolymerization." What other terms apply to the process?*

2 *A positive result for the DNA hydrolysis test does not distinguish between* Staphylococcus *DNase and the DNase produced by* Serratia. *If you had the expertise to correct this weakness in the system, would you be improving the test's sensitivity or its specificity?*

3 *Suggest a reason why this test is read after only 24 hours while other tests (e.g., gelatinase test) may take a week. What would be the likely consequences of incubating DNase agar for a week?*

4 *Why is the uninoculated control sector relatively unnecessary in this test?*

5 *Why is it advisable to use a positive control along with organisms that you are testing?*

6 *In what ways can a DNase (+) organism use the products of DNA hydrolysis?* (**Hint:** *examine Figs. A.1 and A.4.)*

Lipid Hydrolysis (Lipase Test)

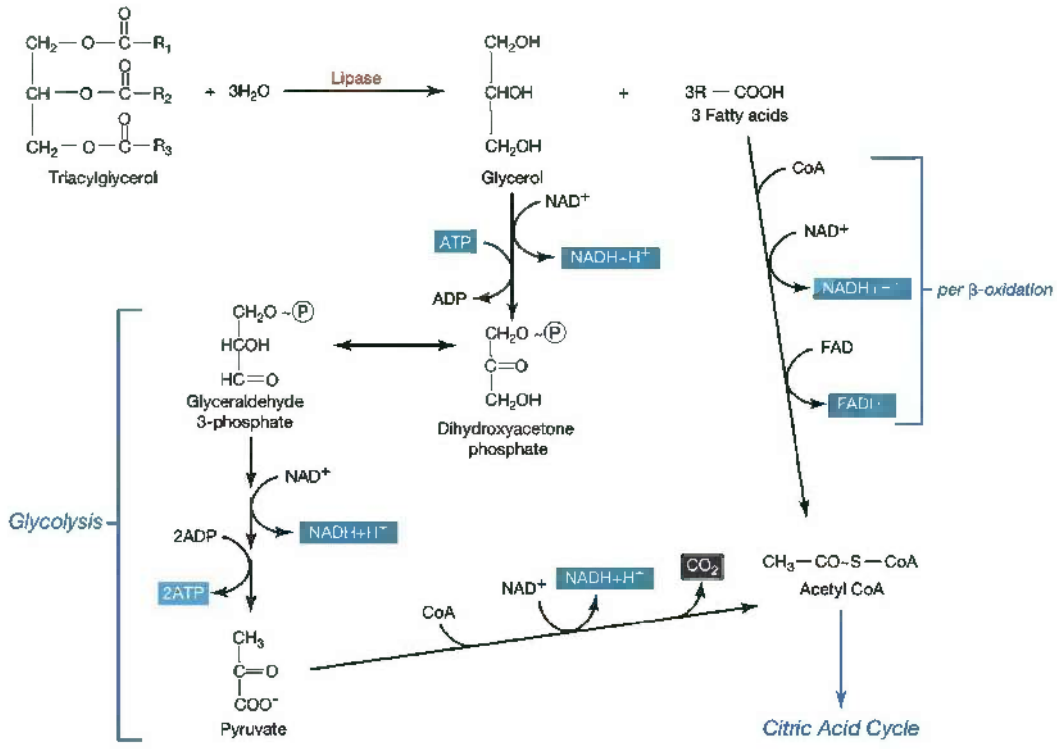
EXERCISE 5-15

Theory

The word **lipid** is generally used to describe all types of fats. Bacterial enzymes that hydrolyze lipids fall into a generic category called **lipases**. Because lipases are secreted by the organism to digest lipids outside the cell, they are called extracellular enzymes, or exoenzymes. Similar in function to other types of exoenzymes (Exercises 5-13, 5-14, and 5-16) lipases are necessary because intact lipids are macromolecules and too large to be transported into the cell. One purpose of this exercise is to demonstrate that bacteria can be differentiated based on their ability to produce and secrete lipases.

Simple fats, known as **triglycerides**, or **triacylglycerols**, are composed of glycerol and three long-chain fatty acids. After lipolysis (lipid hydrolysis), glycerol can be phosphorylated and oxidized in its conversion to dihydroxyacetone phosphate, which is an intermediate of glycolysis (Figs. 5.50 and A.1). The process costs one ATP, but reduces one NAD^+ to $NADH + H^+$. The fatty acids are catabolized by a process called **β-oxidation** in which two carbon fragments are sequentially removed and combined with Coenzyme A to produce acetyl-CoA, one NADH and one $FADH_2$. Acetyl-CoA can then enter the citric acid cycle. Alternatively, glycerol and fatty acids may be used in anabolic pathways.

A variety of simple fats can be used for the lipid hydrolysis (lipase) test, but tributyrin oil is a common choice because it is the simplest triglyceride found in natural fats. Tributyrin agar is prepared as an emulsion that makes the agar appear opaque. When the plate is inoculated with a lipase-positive organism, clear zones will appear around the growth as evidence of lipolytic activity (Fig. 5.51). If no clear zones appear, the organism is lipase-negative. Table 5-16 summarizes the results and interpretations.



5.50 Lipid Catabolism ♦ A triacylglycerol is a simple fat molecule, composed of a three-carbon alcohol (glycerol) bonded to three fatty acid chains (represented by R_1, R_2, and R_3 in this diagram. The products of lipid catabolism can be used in glycolysis (glycerol) and the citric acid cycle (acetyl-CoA).

5.51 Lipid Hydrolysis Test Results ♦ This tributyrin agar plate was inoculated with a lipase-positive (+) organism above and a lipase-negative (−) organism below.

5

Application

The lipase test is used to detect and enumerate lipolytic bacteria, especially in high-fat dairy products. A variety of other lipid substrates, including corn oil, olive oil, and soybean oil, are used to detect differential characteristics among members of *Enterobacteriaceae*, *Clostridium*, *Staphylococcus*, and *Neisseria*. Several fungal species also demonstrate lipolytic ability.

In This Exercise

You will inoculate a tributyrin agar plate with two organisms, one of which will produce a clearing in the medium surrounding the growth.

✓ Materials

Per Student Group

- ☐ One tributyrin agar plate
- ☐ Fresh cultures of:
  - *Bacillus subtilis* or *B. cereus*
  - *Escherichia coli*

Media Recipe

Tributyrin Agar

• Beef extract	3.0 g
• Peptone	5.0 g
• Agar	15.0 g
• Tributyrin oil	10.0 mL
• Distilled or deionized water	1.0 L

pH 5.8–6.2 at 25°C

TABLE 5-16 Lipase Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Clearing in agar around growth	Lipase is present	+
No clearing in agar around growth	Lipase is absent	−

Procedure

Lab One

1. Using a marking pen, divide the plate into three equal sectors. Be sure to mark on the bottom of the plate, not on the lid.
2. Label the plate with the names of the organisms, your name, and the date.
3. Spot inoculate two sectors with the test organisms, leaving the third sector uninoculated as a control.
4. Invert the plate and incubate it aerobically at 35 ± 2°C for 24 to 48 hours.

Lab Two

Examine the plates for clearing around the bacterial growth, and record your results in the table provided on the data sheet, page 373. If no clearing has developed, reincubate for 24 to 48 hours.

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 114 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Haas, Michael J. Chap. 15 in *Compendium of Methods for the Microbiological Examination of Foods*, 4th ed. Frances Pouch Downes and Keith Ito, eds. Washington, DC: American Public Health Association, 2001.

Knapp, Joan S. and Roselyn J. Rice. Page 335 in *Manual of Clinical Microbiology*, 6th ed. Patrick R. Murray, Ellen Jo Baron, Michael A. Pfaller, Fred C. Tenover, and Robert H. Yolken, eds. Washington, DC: ASM Press, 1995.

MacFaddin, Jean F. Page 286 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Zimbro, Mary Jo and David A. Power, eds. Page 521 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-15

Lipid Hydrolysis (Lipase Test)

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-16, page 372, as a guide, record your results and interpretations in the table below.

Organism	Result	(+/−)	Interpretation (include enzyme)
Control sector			

QUESTIONS

1 *Many organisms possessing many different lipases produce positive results on tributyrin agar. Is the inability of this medium to distinguish between these different enzymes a weakness in its specificity or its sensitivity? Explain your answer.*

2 *Why do you think tributyrin agar is prepared in the blender as an emulsion rather than simply stirred as a solution?*

3 *Tributyrin agar has a shelf life of only a few days before it loses its opacity.*

a. *With this in mind, explain the importance of positive and negative controls in this test.*

b. *How would expired tributyrin agar affect the results of lipase (+) and lipase (−) organisms?*

4 *Imagine this situation: Species #1 and Species #2 are unrelated, but each produces an enzyme capable of hydrolyzing soybean oil. How can this be reconciled with the fact that enzymes are highly specific to their substrates?*

Casein Hydrolysis (Casease Test)

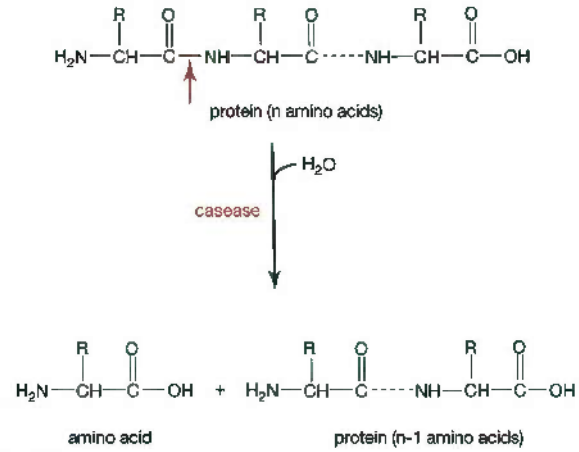
EXERCISE 5-16

Theory

Many bacteria require proteins as a source of amino acids and other components for synthetic processes. Some bacteria have the ability to produce and secrete enzymes into the environment that catalyze the hydrolysis of large proteins to smaller peptides or individual amino acids, thereby enabling their uptake across the membrane (Fig. 5.52).

Casease is an enzyme that some bacteria produce to hydrolyze the milk protein **casein**, the molecule that gives milk its white color. When broken down into smaller fragments, the ordinarily white casein loses its opacity and becomes clear.

The presence of casease can be detected easily with the test medium milk agar (Fig. 5.53). Milk agar is an undefined medium containing pancreatic digest of casein, yeast extract, dextrose, and powdered milk. When plated milk agar is inoculated with a casease-positive organism, secreted casease will diffuse into the medium around the colonies and create a zone of clearing where the casein has been hydrolyzed. Casease-negative organisms do not secrete casease and do not produce clear zones around the growth.



5-52 Casein Hydrolysis ◆ Hydrolysis of any protein occurs by breaking peptide bonds (red arrow) between adjacent amino acids to produce short peptides or individual amino acids.

Application

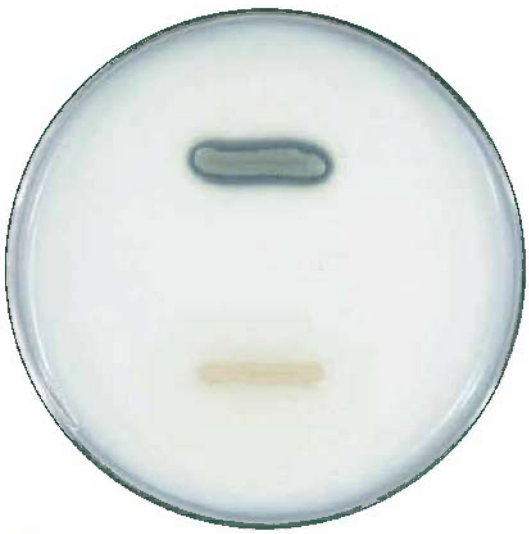
The casein hydrolysis test is used for the cultivation and differentiation of bacteria that produce the enzyme casease.

In This Exercise

You will inoculate a single milk agar plate with a casease-positive and a casease-negative organism. Use Table 5-17 as a guide when making your interpretations.

TABLE 5-17 Casease Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Clearing in agar	Casease is present	+
No clearing in agar	Casease is absent	–



5.53 Casein Hydrolysis Test Results ◆ This milk agar plate was inoculated with a casease-positive (+) organism above and a casease-negative (–) organism below. The clearing around the casease-positive organism is dark because of the background behind the plate, not because it should look that color.

✓ Materials

Per Student Group

- ☐ One milk agar plate
- ☐ Fresh cultures of:
  - *Bacillus subtilis* or *B. cereus*
  - *Escherichia coli*

■ Medium Recipe

Skim Milk Agar

• Pancreatic digest of casein	5.0 g
• Yeast extract	2.5 g
• Powdered nonfat milk	100.0 g
• Glucose	1.0 g
• Agar	15.0 g
• Distilled or deionized water	1.1 L

pH 6.9–7.1 at 25°C

5

Procedure

Lab One

1. Using a marking pen, divide the plate into three equal sectors. Be sure to mark on the bottom of the plate, not the lid.
2. Label the plate with the names of the organisms, your name, and the date.
3. Spot inoculate two sectors with the test organisms and leave the third sector uninoculated as a control.
4. Invert the plate and incubate it aerobically at 35 ± 2°C for 24 hours. *Note*: *Bacillus* species grow very well on milk agar. If you cannot read the plate in 24 hours, move it to the refrigerator or the *Bacillus* may have overgrown your entire plate by the time you get to it.

Lab Two

1. Examine the plates for clearing around the bacterial growth.
2. Record your results in the table provided on the data sheet, page 377.

References

Atlas, Ronald M. Page 1563 in *Handbook of Microbiological Media*, 3rd ed. Boca Raton, FL: CRC Press LLC, 2004.

Holt, John G., ed. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Baltimore: Lippincott Williams & Wilkins, 1994.

Smibert, Robert M. and Noel R. Krieg. Page 613 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Zimbro, Mary Jo and David A. Power, eds. Page 364 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-16

Casein Hydrolysis (Casease Test)

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-17, page 375, as a guide, record your results and interpretations in the table below.

Organism	Result	(+/−)	Interpretation (include enzyme)
Control sector			

QUESTIONS

1 *What does the enzyme casease have in common with amylase (Exercise 5-13)? How are they different?*

2 *How do we know that casease is an exoenzyme and not a cytoplasmic enzyme?*

3 *Consider incubation time for this test.*

a. *Is it acceptable to read a positive test before the incubation time is completed? Explain.*

b. *How about an early negative result? Explain.*

4 *Why is the uninoculated control relatively unnecessary in this test?*

5 *Why is it advisable to use a positive control along with organisms that you are testing?*

Gelatin Hydrolysis (Gelatinase Test)

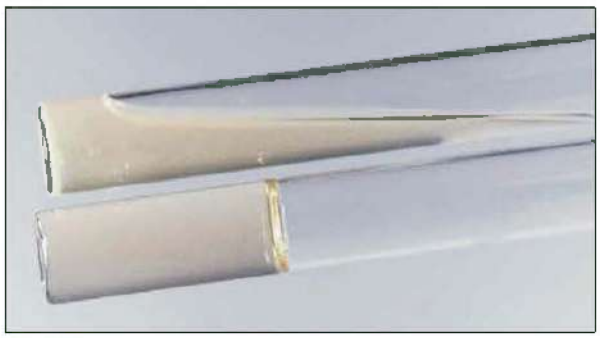
EXERCISE 5-17

Theory

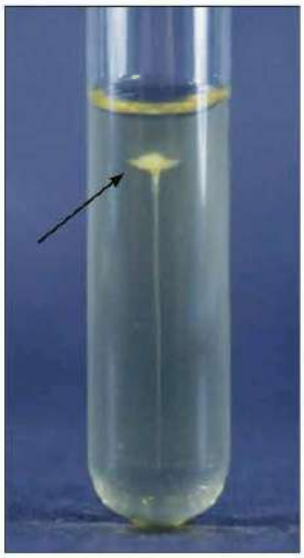
Gelatin is a protein derived from collagen—a component of vertebrate connective tissue. **Gelatinases** comprise a family of extracellular enzymes produced and secreted by some microorganisms to hydrolyze gelatin. Subsequently, the cell can absorb individual amino acids and use them for metabolic purposes. Bacterial hydrolysis of gelatin occurs in two sequential reactions, as shown in Figure 5.54.

The presence of gelatinases can be detected using nutrient gelatin, a simple test medium composed of gelatin, peptone, and beef extract. Nutrient gelatin differs from most other solid media in that the solidifying agent (gelatin) is also the substrate for enzymatic activity. Consequently, when a tube of nutrient gelatin is stab inoculated with a gelatinase-positive organism, secreted gelatinase (or gelatinases) will liquefy the medium. Gelatinase-negative organisms do not secrete the enzyme and do not liquefy the medium (Figs. 5.55 and 5.56). A 7-day incubation period is usually sufficient to see liquefaction of the medium. However, gelatinase activity is very slow in some organisms. All tubes still negative after 7 days should be incubated an additional 7 days.

5.54 Gelatinase Test Results in Nutrient Gelatin ♦ Gelatin is hydrolyzed by the gelatinase family of enzymes.



5.55 Gelatinase Test Results ♦ A gelatinase-positive (+) organism is above, and a gelatinase-negative (−) organism is below. It is not usually necessary to tip the nutrient gelatin tubes as much as is shown in the photo to see whether or not the gelatin is liquid. We exaggerated the angle for the photograph and the tubes had screw caps. Nevertheless, be careful not to spill liquefied gelatin out the top. If this happens, clean up the spill with disinfectant.



5.56 Crateriform Liquefaction ♦ This form of liquefaction also may be of diagnostic use because not all gelatinase-positive microbes liquefy the gelatin completely. Shown here is an organism liquefying the gelatin in the shape of a crater (arrow).

A slight disadvantage of nutrient gelatin is that it melts at 28°C (82°F). Therefore, inoculated stabs are typically incubated at 25°C along with an uninoculated control to verify that any liquefaction is not temperature related.

Application

This gelatin hydrolysis test is used to determine the ability of a microbe to produce gelatinases. *Staphylococcus aureus*, which is gelatinase-positive, can be differentiated from *S. epidermidis*. *Serratia* and *Proteus* species are gelatinase–positive members of *Enterobacteriaceae*, whereas most others in the family are negative. *Bacillus anthracis*, *B. cereus*, and several other members of the genus are gelatinase-positive, as are *Clostridium tetani* and *C. perfringens*.

In This Exercise

You will inoculate two nutrient gelatin tubes and incubate them for up to a week. Gelatin liquefaction is a positive result but is difficult (next to impossible) to differentiate from gelatin that has melted as a result of temperature. Therefore, be sure to incubate an uninoculated control tube along with the others. If the control melts as a result of temperature, refrigerate all tubes until it resolidifies. Use Table 5-18 as a guide to interpretation.

TABLE **5-18** Gelatinase Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Gelatin is liquid (control is solid)	Gelatinase is present	+
Gelatin is solid	No gelatinase is present	–

✓ Materials

Per Student Group

- ☐ Three nutrient gelatin stabs
- ☐ Ice bath or refrigerator
- ☐ Fresh cultures of:
  - *Bacillus subtilis* or *B. cereus*
  - *Escherichia coli*

5

■ Medium Recipe

Nutrient Gelatin

• Beef extract	3.0 g
• Peptone	5.0 g
• Gelatin	120.0 g
• Distilled or deionized water	1.0 L
pH 6.6–7.0 at 25°C	

Procedure

Lab One

1. Obtain three nutrient gelatin stabs. Label each of two tubes with the name of the organism, your name, and the date. Label the third tube "control."
2. Stab inoculate two tubes with heavy inocula of the test organisms. Do not inoculate the control.
3. Incubate all tubes at 25°C for up to 1 week. (A higher incubation temperature (e.g., 35°C) may be used, but an uninoculated control is an absolute necessity.)

Lab Two

1. Examine the control tube. If the gelatin is solid, the test can be read. If it is liquefied, place all tubes in the ice bath or refrigerator until the control has resolidified.
2. When the control has solidified (it shouldn't take more than 15–20 minutes, but it must become solid before proceeding), examine the inoculated media for gelatin liquefaction. Typically, the tubes do not need to be tipped as much as is shown in Figure 5.55. Regardless, be careful not to spill liquefied gelatin out the top when tipping the tubes. If this happens, clean up the culture spill with disinfectant.
3. Record your results in the table provided on the data sheet, page 381.

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 112 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Lányi, B. Page 44 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 128 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Smibert, Robert M. and Noel R. Krieg. Page 617 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Tille, Patricia M. Page 208 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power, eds. Page 408 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-17

Gelatin Hydrolysis (Gelatinase Test)

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-18, page 380, as a guide, record your results and interpretations in the table below.

Organism	Result	(+/−)	Interpretation (include enzyme)
Control			

QUESTIONS

1 *Some microbiologists recommend incubating this medium at 37°C, along with an uninoculated control, and then transferring all tubes to the refrigerator prior to reading them. Why might this be the preferred technique in some situations? What potential problems can you see with this method?*

2 *If the control is solid and an inoculated tube is liquid, is it acceptable to read the result before the complete incubation time has elapsed? Explain.*

3 *If the control is solid and an uninoculated tube is also solid, is it acceptable to read the result before the complete incubation time has elapsed? Explain.*

4 *In the Theory, we mention that some organisms are slow gelatin liquefiers. What about an organism might make it a slow gelatin liquefier?*

5 *Suppose that after 7 days a tube inoculated with a slow liquefier shows no evidence of liquefaction. Is this due to poor sensitivity or poor specificity of the medium? Explain.*

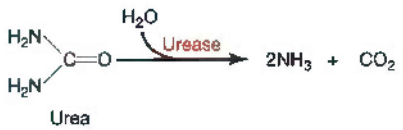
Urea Hydrolysis (Urease Test)

EXERCISE 5-18

Theory

Urea is a product of decarboxylation of certain amino acids and is the primary nitrogenous waste in the urine of many land animals (including mammals). It can be hydrolyzed to ammonia and carbon dioxide by bacteria containing the enzyme **urease** (Fig. 5.57). Urea hydrolysis provides nitrogen in a usable form (ammonia) and it also acts as a virulence factor for some pathogens (e.g., *Helicobacter pylori*) by counteracting acid in the environment. *Streptococcus salivarius*, an oral commensal, also produces urease. Many enteric bacteria possess the ability to metabolize urea, and the urease genes are activated when a usable nitrogen source is absent or urea is present.

Urea agar was formulated to differentiate rapid urease-positive bacteria (e.g., *Proteus*, *Morganella morganii*, and some *Providencia stuartii* strains) from slower urease-positive and urease-negative bacteria. It contains urea, peptone, potassium phosphate, glucose, and phenol red. Peptone and glucose provide essential nutrients for a broad range of bacteria. Potassium phosphate is a mild buffer used to resist alkalinization of the medium from peptone metabolism. Phenol red, which is yellow or orange below pH 8.4 and red or pink above, is included as an indicator.



5.57 Urea Hydrolysis ◆ Urea hydrolysis produces ammonia, which raises the pH in the medium and turns the pH indicator pink. Ammonia can be used by some organisms as a nitrogen source. It also can protect against an acidic environment.

Urea hydrolysis to ammonia by urease-positive organisms will overcome the buffer in the medium and change it from orange to pink. The agar must be examined daily during incubation. Rapid urease-positive organisms will turn the entire slant pink within 24 hours. Weak positives may take several days (Table 5-19). Urease-negative organisms either produce no color change in the medium or turn it yellow from acid products (Fig. 5.58).

Urea broth differs from urea agar in two important ways. First, its only nutrient source is a trace (0.0001%) of yeast extract. Second, it contains buffers strong enough to inhibit alkalinization of the medium by all but the rapid

5.58 Urea Hydroylsis Test Results (Urea Agar) ◆ These urease agar tubes after a 24-hour incubation illustrate a urease-negative organism on the left and a rapid urea splitter (+) on the right. An uninoculated control is in the center.

TABLE **5-19** **Urea Agar Test Results and Interpretations**

Table of Results			
Result			
24 hours	24 hours to 6 days	Interpretation	Symbol
All pink		Rapid urea hydrolysis; strong urease production	+
Partially pink	All pink or partially pink	Slow urea hydrolysis; weak urease production	w+
Orange or yellow	All pink or partially pink	Slow urea hydrolysis; weak urease production	w+
Orange or yellow	Orange or yellow	No urea hydrolysis; urea is absent	–

urease-positive organisms mentioned previously. Phenol red, which is yellow or orange below pH 8.4 and red or pink above, is included to expose any increase in pH. Pink color in the medium in less than 24 hours indicates a rapid urease-positive organism. Orange or yellow is negative (Fig. 5.59 and Table 5-20).

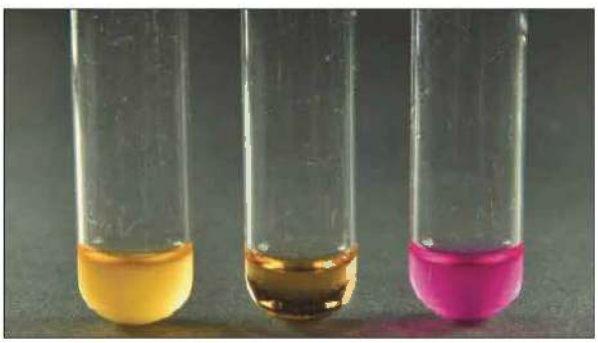
Application

This test is used to differentiate organisms based on their ability to hydrolyze urea with the enzyme urease. Urinary tract pathogens from the genus *Proteus* may be distinguished from other enteric bacteria by their rapid urease activity. It is also used to in identifying *H. pylori*, which is associated with gastric and duodenal ulcers, as well as stomach cancer.

5

In This Exercise

You will perform one or both forms of the test to determine urease activity of three organisms. Urease slants may require incubation up to 6 days, but broths should be read at 24 hours. If your labs are scheduled more than 24 hours apart, arrange to have someone place your broths in a refrigerator until you can examine them.



5.59 Urea Hydroylsis Test Results (Urea Broth) ◆ These urea broth tubes after a 24-hour incubation illustrate a urease-negative organism (−) on the left, an uninoculated control in the middle, and a urease-positive organism (+) on the right.

TABLE 5-20 Urea Broth Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Pink	Rapid urea hydrolysis; strong urease production	+
Orange or yellow	No urea hydrolysis; organism does not produce urease or cannot live in broth	−

✓ Materials

Per Student Group

- □ Four urea broths
- □ Four urea agar slants
- □ Six sterile wooden sticks or sterile disposable loops
- □ Fresh slant cultures of:
  - *Escherichia coli*
  - *Klebsiella pneumoniae* (BSL-2)[1]
  - *Proteus vulgaris* (BSL-2)

Medium Recipes

Christensen's Urea Agar

• Peptone	1.0 g
• Dextrose (glucose)	1.0 g
• Sodium chloride	5.0 g
• Potassium phosphate, monobasic	2.0 g
• Urea	20.0 g
• Agar	15.0 g
• Phenol red	0.012 g
• Distilled or deionized water	1.0 L
pH 6.8 at 25°C	

Rustigian and Stuart's Urea Broth

• Yeast extract	0.1 g
• Potassium phosphate, monobasic	9.1 g
• Potassium phosphate, dibasic	9.5 g
• Urea	20.0 g
• Phenol red	0.01 g
• Distilled or deionized water	1.0 L
pH 6.8 at 25°C	

Procedure

Lab One

1. Obtain four tubes of each medium. Label three with the names of the organisms, your name, and the date. Label the fourth tube of each medium "control."
2. Inoculate three urea broths with heavy inocula from the test organisms. **Use BSL-2 precautions for *P. vulgaris* and *K. pneumoniae*,** and dispose of the sterile sticks/disposable loops according to your lab's practices. Do not inoculate the control.

[1] The most consistently urease-positive organisms are the ones listed. If the goal of this exercise is simply to show a positive urease result (and not rapid vs. slow urease positives), *Staphylococcus epidermidis* and *S. saprophyticus* are BSL-1 organisms that are generally urease positive. Adjust the number of tubes accordingly.

3. Streak inoculate three urea slants with the test organisms, covering the entire agar surface with a heavy inoculum. **Use BSL-2 precautions for *P. vulgaris* and *K. pneumoniae***, and dispose of the sterile sticks/disposable loops properly. Do not stab the agar butt. Do not inoculate the control.
4. Incubate all tubes aerobically at 35 ± 2°C for 24 hours. (If your labs are scheduled more than 1 day apart, try to read these media at 24 hours or arrange to have someone else read them for you. Positive results can be removed and placed in the refrigerator for later examination.)

Lab Two

1. Remove all tubes from the incubator and examine them for color changes. Record all broth results and any rapid positive and slow positive agar test results in the tables provided on the data sheet, page 387. Discard all broth tubes and positive agar tubes in an appropriate autoclave container.
2. Return any negative agar tubes to the incubator. Inspect them daily for pink color formation for up to 6 days. (Negative tubes must not be recorded as negative until they have incubated the full 6 days.)
3. Enter your agar results daily in the table provided. Refer to Tables 5-19 and 5-20 when interpreting your results.

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 117 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Delost, Maria Dannessa. Page 196 in *Introduction to Diagnostic Microbiology*. St. Louis, MO: Mosby, 1997.

DIFCO Laboratories. Page 1040 in *DIFCO Manual*, 10th ed. Detroit: DIFCO Laboratories, 1984.

Forbes, Betty A., Daniel F. Sahm, and Alice S. Weissfeld. Page 283 in *Bailey & Scott's Diagnostic Microbiology*, 11th ed. St. Louis, MO: Mosby, 2002.

Lányi, B. Page 24 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 298 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Moat, Albert G., John W. Foster, and Michael P. Spector. Pages 491–492 in *Microbial Physiology*, 4th ed. New York: Wiley-Liss, 2002.

Smibert, Robert M. and Noel R. Krieg. Page 630 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Tille, Patricia M. Page 229 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckenberger, and Gail Woods. Pages 225 and 1458–1459 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Zimbro, Mary Jo and David A. Power, eds. Page 606 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-18

Urea Hydrolysis (Urease Test)

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-19, page 383, as a guide, enter your results for the agar test each day for 6 days or until pink color appears.

Urea Agar								
	Color							
Organism	24 Hrs.	2 Days	3 Days	4 Days	5 Days	6 Days	+/−	Interpretation (include enzyme)
Control								

2 Using Table 5-20, page 384, as a guide, enter your results for the broth test after 24 hours.

Urea Broth			
Organism	Color Result	+/−	Interpretation (include enzyme)
Control			

QUESTIONS

1 *Suppose you ran this test with* Providencia stuartii *but it took 48 hours to turn pink.*

a. *Do you think this is a false result?*

b. *If so, is it a false positive or a false negative? If not, why not? Give some possible reasons for this occurrence.*

2 *Explain why it is acceptable to record positive tests before the suggested incubation time is completed but it is not acceptable to record a negative result early.*

3 *Typically, urea broth is filter-sterilized because autoclaving breaks down the urea. However, even unsterilized broth rarely produces false-positive results. Why do you think this is true?*

4 *Compare the tests.*

a. *Did your results from the broth and solid media agree for each organism?*

b. *Did you expect them to? Why or why not?*

c. *Explain any differences.*

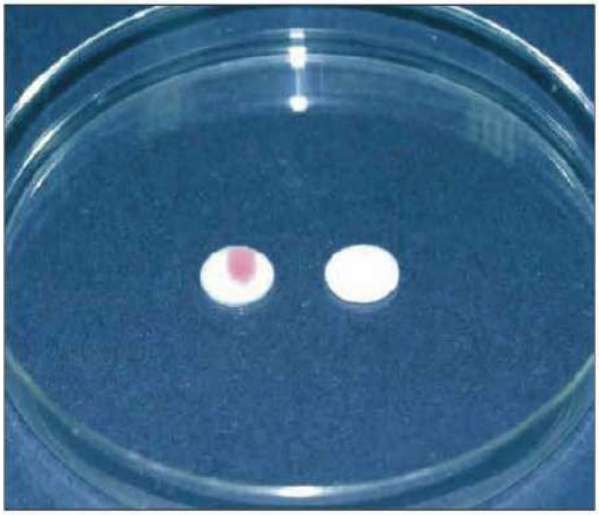
EXERCISE
5-19

PYR Test

Theory

Aminopeptidases are enzymes that remove amino acids from the amino end of a protein or peptide. A specific example is L-pyrrolidonyl arylamidase, which is produced by group A streptococci (*S. pyogenes*) and enterococci. This enzyme hydrolyzes L-pyrrolidonyl–β–naphthylamide (PYR) to produce L-pyrrolidone and β-naphthylamine, all of which are colorless. In the indicator reaction, β-naphthylamine reacts with *p*-dimethylaminocinnamaldehyde to form a red precipitate (Fig. 5.60).

PYR may be performed as an 18-hour agar test, a four-hour broth test or, as used in this example, a rapid disk test. In each case the medium (or disk) contains pyroglutamyl-β-naphthylamide to which is added a heavy inoculum of the test organism. After the appropriate incubation or waiting period, a 0.01% *p*-dimethylaminocinnamaldehyde solution is added. Formation of a red color within a few minutes is interpreted as PYR-positive (Fig. 5.61). Yellow or orange is PYR-negative. Refer to Table 5-21 as a guide to interpretation.



5.61 PYR Disk Test Results ◆ The disk on the left was inoculated with a PYR-positive organism; the disk on the right was inoculated with a PYR–negative organism.

Application

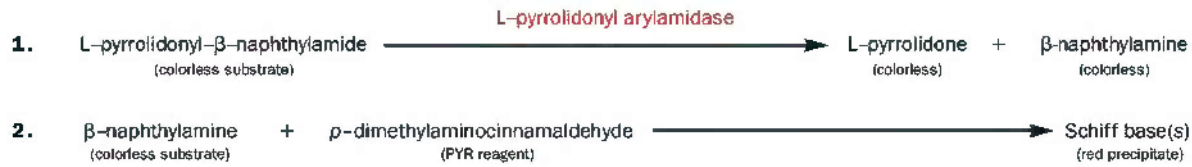
The PYR test is designed for presumptive identification of group A streptococci (*S. pyogenes*) and enterococci by determining the presence of the enzyme L-pyrrolidonyl arylamidase.

In This Exercise

You will inoculate two disks with test organisms to determine which one contains the enzyme L-pyrrolidonyl arylamidase.

TABLE **5-21** **PYR Test Results and Interpretations**

Table of Results		
Result	**Interpretation**	**Symbol**
Red color formation	Organism produces L-pyrrolidonyl arylamidase	+
Orange color or no color change	Organism does not produce L-pyrrolidonyl arylamidase	–



5.60 PYR Chemistry ◆ Reaction 1 shows the hydrolytic splitting of the substrate L–pyrrolidonyl–β–naphthylamide included in the medium. In this exercise, the filter-paper disks were saturated with substrate. Both products of the reaction are colorless. Reaction 2 illustrates the reaction of the PYR reagent (*p*-dimethylaminocinnamaldehyde) and β–naphthylamine. In the reaction, the amino group (H_2N) of β–naphthylamine reacts with the aldehyde group (–CH_4O) of the reagent and produces a Schiff base (–CH4N–), which is red.

✓ Materials

Per Student Group

- ☐ One empty Petri dish or glass slide
- ☐ PYR disks
- ☐ 0.01% *p*-dimethylaminocinnamaldehyde
- ☐ Fresh slant cultures of:
  - *Enterococcus faecalis* (BSL-2)
  - *Staphylococcus epidermidis*

Procedure

1. Obtain a Petri dish or glass slide, three PYR disks, and two sterile pipettes.
2. Open the Petri dish and mark three spots where you will place the PYR disks. Label two spots with the name of its respective organism. Label the third spot "control."
3. Place three PYR disks inside on the appropriate spots.
4. With a disposable loop or sterile wooden stick, inoculate each disk with a large amount of its respective organism. Properly dispose of the loop/wooden stick immediately after use. **Use extra caution with *E. faecalis*, because it is BSL-2.**
5. Add a drop of the *p*-dimethylaminocinnamaldehyde solution to each disk.
6. If the disk turns red within 5 minutes, it is positive. Either orange or no color change are negative results.
7. Record your results in the table on the data sheet, page 391.

References

Claus, G. William. Chap. 14 in *Understanding Microbes—A Laboratory Textbook for Microbiology*. New York: W.H. Freeman and Company, 1989.

Collins, C. H., Patricia M. Lyne and J. M. Grange. Chap. 6 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heineman, 1995.

Tille, Patricia M. Pages 97 and 225 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014

Winn, Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckenberger, and Gail Woods. Page 1448 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-19

PYR Test

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-21, page 389, as a guide, record your results and interpretations in the table below.

Organism	Result	(+/−)	Interpretation (include enzyme)

QUESTIONS

1 *Consider the uninoculated disk.*

a. *Is it a positive or a negative control?*

b. *What purpose does it serve in the PYR test?*

2 *How are the products of protein hydrolysis by an aminopeptidase used in metabolism?*

3 *Deep red is the only color reaction that is considered positive for this test. Why do you think yellow or orange results are not positive (or weak positive)?*

4 *Suppose you wanted to perform this test on an unknown organism but you were out of PYR reagent. Suppose further that a fellow lab employee said that you could substitute formaldehyde for PYR reagent and get good results. What is the chemical basis for your peer's suggestion?*

Combination Differential Media

Combination differential media combine components of several compatible tests into one medium, thus saving critical diagnostic time and money. Typically, they include core tests to differentiate members of specific bacterial groups. SIM medium, for example, tests for sulfur reduction, indole production, and motility—important characteristics of members of *Enterobacteriaceae*.

As with most biochemical tests, combination media can be used as a follow up to selective media. Selective media promote isolation of the organisms of interest and help determine the sequence of tests to follow. MacConkey agar (Exercise 4-5), for example, may be streaked initially to encourage and isolate Gram-negative organisms, followed by Kligler iron agar (KIA). KIA tests for glucose and lactose fermentation and for sulfur reduction, diagnostically important tests for many Gram-negative organisms. ◆

SIM Medium Sulfur Reduction, Indole Production, Motility

EXERCISE 5-20

5

Theory

SIM medium is used for determination of three bacterial activities: sulfur reduction, indole production from tryptophan, and motility (from which the acronym "SIM" is derived). The semisolid medium includes casein and animal tissue as sources of amino acids, an iron-containing compound, and sulfur in the form of sodium thiosulfate.

Sulfur reduction to H_2S can be accomplished by bacteria in two different ways, depending on the enzymes present.

1. The enzyme **cysteine desulfurase** catalyzes the hydrolysis of the sulfur-containing amino acid cysteine to pyruvate and H_2S during putrefaction (Fig. 5.62).
2. In a totally unrelated way, the enzyme **thiosulfate reductase** catalyzes the reduction of sulfur (in the form of sulfate) to H_2S at the end of an anaerobic respiratory electron transport chain (Fig. 5.63).

Both systems produce hydrogen sulfide (H_2S) gas. When either reaction occurs in SIM medium, the H_2S produced combines with iron, in the form of ferrous ammonium sulfate, to form ferric sulfide (FeS), a black precipitate (Fig. 5.64). Any blackening of the medium is an indication of sulfur reduction and a positive test. No blackening of the medium indicates no sulfur reduction and a negative reaction (Fig. 5.65).

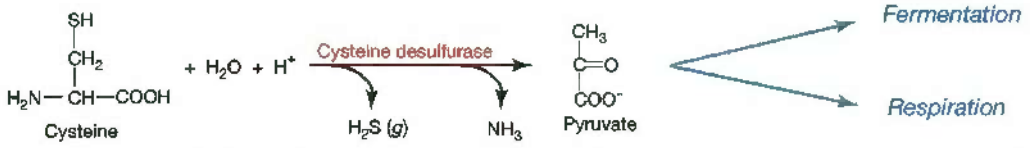
Indole production in the medium is made possible by the presence of tryptophan (contained in casein and animal protein). Bacteria possessing the enzyme **tryptophanase** can hydrolyze tryptophan to pyruvate, ammonia (by deamination), and indole (Fig. 5.66).

$$3S_2O_3^{=} + 4H^+ + 4e^- \xrightarrow{\text{Thiosulfate reductase}} 2SO_3^{=} + 2H_2S\,(g)$$

5.63 Sulfur Reduction: H_2S Production from Anaerobic Respiration (Reduction of Thiosulfate) ◆ Anaerobic respiration with thiosulfate as the final electron acceptor also produces H_2S.

$$H_2S + FeSO_4 \longrightarrow H_2SO_4 + FeS\,(s)$$

5.64 Indicator Reaction for H_2S Production ◆ Hydrogen sulfide, a colorless gas, can be detected when it reacts with ferrous sulfate in the medium to produce the black precipitate ferric sulfide.

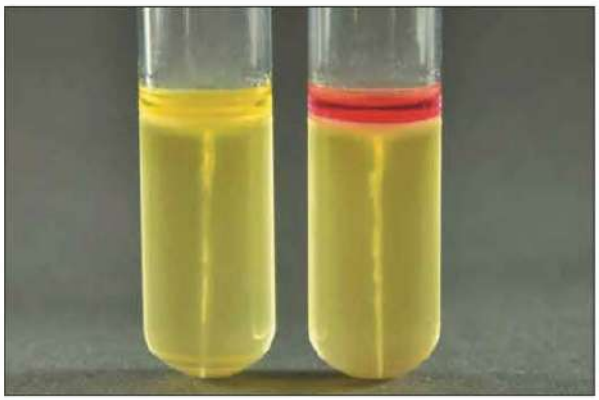


5.62 Sulfur Reduction: H_2S Production from Cysteine Hydrolysis (Putrefaction) ◆ Cysteine desulfurase catalyzes the hydrolysis of cysteine with the production of pyruvate, NH_3, and H_2S. This reaction is a mechanism for getting energy out of the amino acid cysteine.

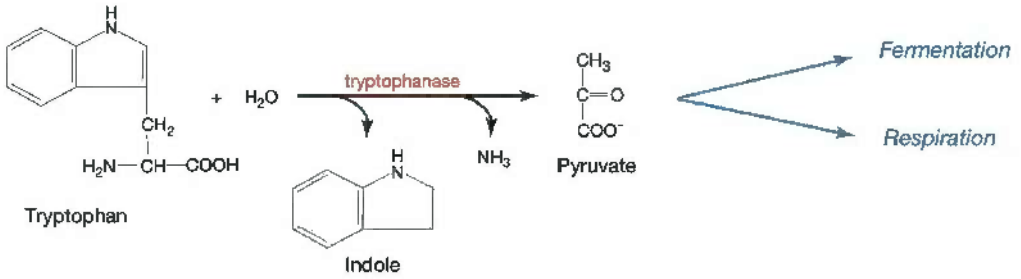
Tryptophan hydrolysis in SIM medium can be detected by the addition of Kovac's reagent after a period of incubation. Kovac's reagent contains ***p*-dimethylamino-benzaldehyde** (DMABA) and HCl dissolved in amyl alcohol. When a few drops of Kovac's reagent are added to the tube, DMABA reacts with any indole present and produces a quinoidal compound that turns the reagent layer red (Figs. 5.67 and 5.68). The formation of red color in the reagent layer indicates a positive reaction and the presence of tryptophanase. No red color is indole negative.

Determination of motility in SIM medium is made possible by the reduced agar concentration and the method of inoculation. The medium is inoculated with a single stab from an inoculating needle. Motile organisms are able to move about in the semisolid medium and can be detected by the radiating growth extending outward in all directions from the central stab line. Growth that radiates in *all* directions and appears slightly fuzzy is an indication of motility (Fig. 5.69). This should not be confused with the (seemingly) spreading growth produced by lateral movement of the inoculating needle when stabbing.

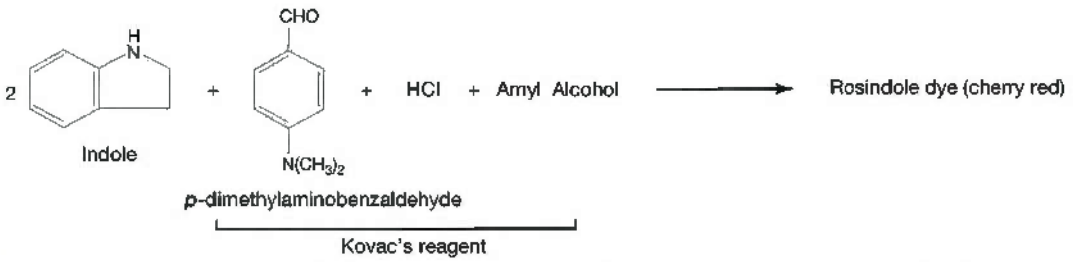
5.65 Sulfur Reduction in SIM Medium ♦ The organism on the left is H_2S negative. The organism on the right is H_2S positive. Although without a control it is difficult to tell, the organism on the left is motile (note the haziness in the medium). When an organism is H_2S positive it is nearly impossible to read motility in this medium.



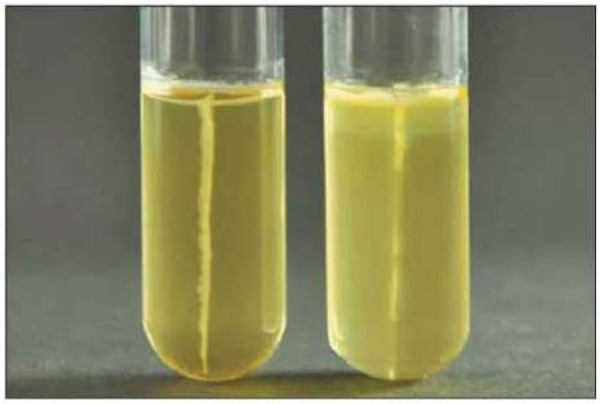
5.68 Indole Test Results ♦ These SIM tubes were inoculated with an indole-negative (−) organism on the left and an indole-positive (+) organism on the right. Note how little Kovac's reagent is used. Both organisms are also motile.



5.66 Tryptophan Catabolism in Indole-Positive Organisms ♦ This reaction is a mechanism for getting energy out of the amino acid tryptophan; the enzyme responsible is tryptophanase.



5.67 Indicator Reaction for Indole Production with Kovac's Reagent ♦ If present, indole reacts with the DMABA in Kovac's reagent to produce a red color.



5.69 Motility in SIM ◆ These SIM tubes were inoculated with a nonmotile organism on the left and a motile organism on the right. With an adequate control, the haziness in the agar indicating motility is usually detectable. However, if results are equivocal, try motility agar (Exercise 5-28).

Application

SIM medium is used to identify bacteria that are capable of producing indole, using the enzyme tryptophanase. The indole test is one component of the IMViC battery of tests (*I*ndole, *M*ethyl red, *V*oges-Proskauer, and *C*itrate) used to differentiate the *Enterobacteriaceae*, especially *E. coli* (+) from *Enterobacter*, *Klebsiella*, *Hafnia*, and *Serratia* (–). SIM medium also is used to differentiate sulfur-reducing members of *Enterobacteriaceae*, especially members of the genera *Salmonella*, *Shigella*, and *Proteus* from the negative *Morganella morganii* and *Providencia rettgeri*. In addition to the first two functions of SIM, motility is an important differential characteristic of *Enterobacteriaceae*.

In This Exercise

You will stab inoculate SIM medium with three organisms demonstrating a variety of results. When reading your test results, make the motility and H_2S determinations before adding the indole reagent. Use Tables 5-22, 5-23 and 5-24, and Figures 5.65, 5.68, and 5.69 as guides when making your interpretations.

TABLE 5-22 Sulfur Reduction Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Black in the medium	Sulfur reduction (H_2S production)	+
No black in the medium	Sulfur is not reduced	–

TABLE 5-23 Indole Production Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Red in the alcohol layer of Kovac's reagent	Organism produces tryptophanase and hydrolyzes tryptophan into indole and pyruvate	+
Reagent color is unchanged	Organism doesn't produce tryptophanase and tryptophan is not hydrolyzed	–

TABLE 5-24 Motility Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Growth radiating outward from stab line	Motility	+
No radiating growth	Nonmotile	–
Blackened medium (H_2S produced)	Motility not determined	ND

Materials

Per Student Group

- ☐ Four SIM tubes
- ☐ Kovac's reagent
- ☐ Microbial incinerator or disposable inoculating needle(s)
- ☐ Fresh cultures of:
  - *Escherichia coli*
  - *Micrococcus luteus*
  - *Proteus mirabilis* (BSL-2)[1]

[1] Approximately 75% of *Citrobacter freundii* strains are positive for sulfur reduction and it may serve as a suitable BSL-1 replacement for *P. mirabilis*, if desired. *Aeromonas hydrophila* may also work as a substitute. Check your lab's strains prior to assigning these.

■ Medium and Reagent Recipes

SIM (Sulfur-Indole-Motility) Medium

• Pancreatic digest of casein	20.0 g
• Peptic digest of animal tissue	6.1 g
• Ferrous ammonium sulfate	0.2 g
• Sodium thiosulfate	0.2 g
• Agar	3.5 g
• Distilled or deionized water	1.0 L

pH 7.1–7.4 at 25°C

Kovac's Reagent

• Amyl alcohol	75.0 mL
• Hydrochloric acid, concentrated	25.0 mL
• *p*-dimethylaminobenzaldehyde	5.0 g

Procedure

5

Lab One

1. Obtain four SIM tubes. Label three with the names of the organisms, your name, and the date. Label one tube "control."
2. Stab inoculate three tubes with the test organisms. Insert the needle into the agar to within 1 cm of the bottom of the tube. Be careful to remove the needle along the original stab line. Do not inoculate the control. ***Note*: If using *P. mirabilis*, BSL-2 precautions should be observed.** Inoculation can be done with a wire loop if sterilized with a microbial incinerator. Otherwise, a disposable inoculating needle should be used.
3. Incubate all tubes aerobically at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Examine the tubes for spreading from the stab line *and* formation of black precipitate in the medium. Record any H_2S production and/or motility in the table provided on the data sheet, page 397.
2. Add Kovac's reagent to each tube to a depth of 2 mm–3 mm (*not mL*). After several minutes, observe for the formation of red color in the reagent layer.
3. Record your results in the table on the data sheet.

References

Delost, Maria Dannessa. Page 186 in *Introduction to Diagnostic Microbiology*. St. Louis, MO: Mosby, 1997.

MacFaddin, Jean F. Page 162 in *Biochemical Tests for Identification of Medical Bacteria*, 2nd ed. Baltimore: Lippincott Williams & Wilkins, 1980.

Tille, Patricia M. Pages 97 and 210 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckenberger, and Gail Woods. Pages 224, 226, and 1445–1446 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Zimbro, Mary Jo and David A. Power, eds. Page 490 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-20

SIM Medium
Sulfur Reduction, Indole Production, Motility

OBSERVATIONS AND INTERPRETATIONS

1 Using Table 5-22, page 395, as a guide, record your results and interpretations in the table below.

Sulfur Reduction			
Organism	**Black PPT? (Y/N)**	**+/−**	**Interpretation** (include enzyme)
Uninoculated control			

2 Using Table 5-23, page 395, as a guide, record your results and interpretations in the table below.

Indole Reduction			
Organism	**Red Color? (Y/N)**	**+/−**	**Interpretation** (include enzyme)
Uninoculated control			

3 Using Table 5-24, page 395, as a guide, record your results and interpretations in the table below.

Motility			
Organism	**Growth Pattern**	**+/−**	**Interpretation** (include enzyme)
Uninoculated control			

QUESTIONS

1 *Consider the uninoculated SIM tube.*

a. *Is it a positive or a negative control in each test?*

b. *What purpose does it serve in the sulfur reduction test?*

c. *What purpose does it serve in the indole test?*

d. *What purpose does it serve in the motility test?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-20

(continued)

2 *The sulfur reduction test is not able to differentiate H_2S produced by anaerobic respiration and H_2S produced by putrefaction. Is this inability the result of poor sensitivity or poor specificity of the test system?*

3 *What factors dictate the choice of tests included in a combination medium?*

4 *Which ingredient(s) could be eliminated if this medium were used strictly for testing indole production? Explain.*

Triple Sugar Iron Agar/ Kligler Iron Agar[1]

EXERCISE 5-21

Theory

Triple sugar iron agar (TSIA) is a rich medium designed to differentiate bacteria on the basis of glucose fermentation, lactose fermentation, sucrose fermentation, and sulfur reduction. In addition to the three carbohydrates, it includes animal proteins as sources of carbon and nitrogen, and both ferrous sulfate and sodium thiosulfate as sources of oxidized sulfur. Phenol red is the pH indicator, and the iron in the ferrous sulfate is the hydrogen sulfide indicator.

The medium is prepared as a shallow agar slant with a deep butt, thereby providing both aerobic and anaerobic growth environments. It is inoculated by a stab in the agar butt followed by a fishtail streak of the slant. The incubation period is 18 to 24 hours for carbohydrate fermentation and up to 48 hours for hydrogen sulfide reactions. Many reactions in various combinations are possible (Fig. 5.70 and Table 5-25).

When TSIA is inoculated with a glucose-only fermenter, acid products lower the pH and turn the entire medium yellow within a few hours. Because glucose is in short supply (0.1%), it will be exhausted within about 12 hours. As the glucose diminishes, the organisms located in the aerobic region (slant) will begin to break down available amino acids, producing NH_3 and raising the pH. This process, which takes 18 to 24 hours to complete, is called a **reversion** and only occurs in the slant because of the anaerobic conditions in the butt. Thus, a TSIA with a red slant and yellow butt after a 24-hour incubation period indicates that the organism ferments glucose but not lactose and/or sucrose.

Organisms that are able to ferment glucose *and* lactose *and/or* sucrose also turn the medium yellow throughout. However, because the lactose and sucrose concentrations are 10 times higher than that of glucose, resulting in greater acid production for a longer period of time, both slant and butt will remain yellow after 24 hours. Therefore, a TSIA with a yellow slant and butt at 24 hours indicates that the organism ferments glucose and one or both of the other sugars. Gas produced by fermentation of any of the carbohydrates will appear as fissures in the medium or will lift the agar off the bottom of the tube.

Hydrogen sulfide (H_2S) may be produced by the reduction of thiosulfate in the medium or by the breakdown of cysteine in the peptone (Figs. 5.62 and 5.63). Ferrous sulfate reacts with the H_2S to form a black precipitate, usually seen in the butt (Fig. 5.64). Acid conditions must exist for thiosulfate reduction; therefore, black precipitate in the medium is an indication of sulfur reduction *and* fermentation. If the black precipitate obscures the color of the butt, the color of the slant determines which carbohydrates have been fermented (i.e., red slant = glucose fermentation, yellow slant = glucose and lactose/sucrose fermentation).

An organism that does not ferment any of the carbohydrates but utilizes peptone and amino acids will alkalinize the medium and turn it red. If the organism can use the peptone aerobically and anaerobically, both the slant and butt will appear red. An obligate aerobe will turn only the slant red. (*Note*: The difference between a red butt and a butt unchanged by the organism may be

5.70 Results in Kligler Iron Agar Slants ◆ These KIA slants from left to right illustrate: a kaline slant/acid butt (K/A); alkaline slant/no change in the butt (K/NC); uninoculated control; alkaline slant/acid butt, hydrogen sulfide present (K/A, H_2S); and acid slant/acid butt, gas (A/A, G). TSIA tubes with the same appearances would be scored the same. Refer to Table 5-25 for interpretations.

[1] This exercise is written for either triple sugar iron agar (TSIA) or Kligler iron agar (KIA). In form and function the two media are virtually identical except that KIA does not include sucrose. The results of both media look exactly the same. In results indicating fermentation of more than one carbohydrate, TSIA is reported as *glucose and lactose and/or sucrose fermentation*; KIA (because it contains no sucrose) is reported as *glucose and lactose fermentation*. To avoid the awkwardness of repeatedly including both names, we refer only to TSIA. If you are using KIA, simply substitute the name where applicable and do not expect results for sucrose fermentation.

subtle; therefore, comparison with an uninoculated control is always recommended.)

Not surprisingly, timing is critical when reading results. An early reading could reveal yellow throughout the medium, leading you to believe that the organism ferments more than one sugar when it simply has not yet exhausted the glucose. A reading after all of the sugars have been depleted could reveal a yellow butt and red slant, leading you to falsely believe that the organism is a glucose-only fermenter. The timing for interpreting sulfur reduction is not as critical, so tubes that have been interpreted for carbohydrate fermentation can be re-incubated for 24 hours before final H_2S determination is made. Refer to Table 5-25 for information on the correct symbols and method of reporting the various reactions.

Application

5

TSIA and KIA are primarily used to differentiate members of *Enterobacteriaceae* and to distinguish them from other Gram-negative rods such as *Pseudomonas aeruginosa*.

In This Exercise

Today, you will inoculate four TSIA or KIA slants. Use a large inoculum and try not to introduce excessive air when stabbing the agar butt. Also be sure to remove all tubes from the incubator after no more than 24 hours to take fermentation readings.

✓ Materials

Per Student Group

- ☐ Five TSIA or KIA slants
- ☐ Microbial incinerator and wire inoculating needle, or sterile wooden sticks, or disposable inoculating needle if using BSL-2 organisms
- ☐ Recommended organisms (grown on solid media)[2]:
  - *Escherichia coli*
  - *Morganella morganii* (BSL–2)
  - *Pseudomonas aeruginosa* (BSL–2)
  - *Salmonella typhimurium* (BSL–2)

Media Recipes

Triple Sugar Iron Agar

• Pancreatic digest of casein	10.0 g
• Peptic digest of animal tissue	10.0 g
• Dextrose (glucose)	1.0 g
• Lactose	10.0 g
• Sucrose	10.0 g
• Ferrous ammonium sulfate	0.5 g
• Sodium chloride	5.0 g

[2] If your lab is not equipped to handle BSL-2 organisms, these will be adequate substitutes for the BSL-2 organisms recommended: *Alcaligenes faecalis*, *Citrobacter freundii* (Hardy Diagnostics #0315P), and *Citrobacter koseri* (Hardy Diagnostics #0106P).

TABLE **5-25** **TSIA/KIA Results and Interpretations**

Table of Results		
Result	**Interpretation**	**Symbol**
Yellow slant/yellow butt—KIA	Glucose and lactose fermentation with acid accumulation in slant and butt	A/A
Yellow slant/yellow butt—TSIA	Glucose and lactose and/or sucrose fermentation with acid accumulation in slant and butt	A/A
Red slant/yellow butt	Glucose fermentation with acid production; proteins catabolized aerobically (in the slant) with alkaline products (reversion)	K/A
Red slant/red butt	No fermentation; peptone catabolized aerobically and anaerobically with alkaline products; not from *Enterobacteriaceae*	K/K
Red slant/ no change in the butt	No fermentation; peptone catabolized aerobically with alkaline products; not from *Enterobacteriaceae*	K/NC
No change in slant/ no change in butt	Organism is growing slowly or not at all; not from *Enterobacteriaceae*	NC/NC
Black precipitate in the agar	Sulfur reduction (an acid condition, from fermentation of glucose or lactose, exists in the butt even if the yellow color is obscured by the black precipitate)	H_2S
Cracks in or lifting of agar	Gas production	G

- Sodium thiosulfate 0.2 g
- Agar 13.0 g
- Phenol red 0.025 g
- Distilled or deionized water 1.0 L

pH 7.1–7.5 at 25°C

Kligler Iron Agar

- Pancreatic digest of casein 10.0 g
- Peptic digest of animal tissue 10.0 g
- Lactose 10.0 g
- Dextrose (glucose) 1.0 g
- Ferric ammonium citrate 0.5 g
- Sodium chloride 5.0 g
- Sodium thiosulfate 0.5 g
- Agar 15.0 g
- Phenol red 0.025 g
- Distilled or deionized water 1.0 L

pH 7.2–7.6 at 25°C

Procedure

Lab One

1. Obtain five slants. Label four of the slants with the names of the organisms, your name, and the date. Label the fifth slant "control."
2. **Using BSL–2 precautions,** heavily inoculate four slants with the test organisms. First stab the agar butt and then streak the slant. Do not inoculate the control. Be sure to properly dispose of the wooden stick/inoculating needle (if used).
3. Incubate all slants aerobically at 35 ± 2°C for 18–24 hours (no more or less).

Lab Two

Examine the tubes for characteristic color changes and gas production. Use Table 5-25 as a guide while recording your results on the data sheet, page 405. The proper format for recording results is: slant reaction/butt reaction, gas production, hydrogen sulfide production. For example, a tube showing yellow slant, yellow butt, fissures in or lifting of the agar, and black precipitate would be recorded as: A/A, G, H_2S.

References

Delost, Maria Dannessa. Pages 184–185 in *Introduction to Diagnostic Microbiology.* St. Louis, MO: Mosby, 1997.

MacFaddin, Jean F. Page 239 in *Biochemical Tests for Identification of Medical Bacteria,* 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Tille, Patricia M. Page 228 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckenberger, and Gail Woods. Pages 216–217 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology,* 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Zimbro, Mary Jo and David A. Power, eds. Page 283 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media.* Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-21

Triple Sugar Iron Agar / Kligler Iron Agar

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 5-25, page 402, when recording and interpreting your results.

Organism	Color and Gas Result	Symbol	Interpretation (include enzyme)
Control			

QUESTIONS

1 *As mentioned in Theory, the fermentation readings with TSIA and KIA must take place between 18 and 24 hours after inoculation.*

a. *Why is this true?*

b. *Is timing as critical with H_2S readings? Why or why not?*

2 *You learned in Theory that if the black precipitate obscures the color of the butt it must be acidic and scored as "A." Why do you think this is true?* ***Hints:*** *See Figure 5.64 and examine the starting pH of TSIA).*

3 *TSIA and KIA are complex media with many ingredients. What would be the consequences of the following mistakes in preparing this medium? Consider each independently.*

a. *1% glucose is added rather than the amount specified in the recipe.*

b. *Ferrous ammonium sulfate (or ferric ammonium citrate in KIA) is omitted.*

c. *Casein and animal tissue are omitted.*

d. *Sodium thiosulfate is omitted.*

e. *Phenol red is omitted.*

f. *The initial pH is 8.2.*

g. *The agar butt is shallow rather than deep.*

EXERCISE
5-22

Lysine Iron Agar

Theory

Lysine iron agar (LIA) is a combination medium that detects bacterial ability to decarboxylate or deaminate lysine and to reduce sulfur. It contains peptone and yeast extract to support growth, the amino acid lysine for deamination and decarboxylation reactions, and sodium thiosulfate—a source of reducible sulfur. A small amount of glucose (0.1%) is included as a fermentable carbohydrate. Ferric ammonium citrate is included as a sulfur reduction indicator and bromocresol purple is the pH indicator. Bromocresol purple is purple at pH 6.8 and yellow at or below pH 5.2.

LIA is prepared as a slant with a deep butt. This results in an aerobic zone in the slant and an anaerobic zone in the butt. After it is inoculated with two stabs of the butt and a fishtail streak of the slant, the tube is tightly capped and incubated for 18 to 24 hours.

If the medium has been inoculated with a lysine decarboxylase-positive organism, acid production from glucose fermentation will induce production of decarboxylase enzymes. The acidic pH will turn the medium yellow, but subsequent decarboxylation of lysine will produce the amine cadaverine and alkalinize the agar and return it to purple (Fig. 5.38). Purple color throughout indicates lysine decarboxylation. Purple color in the slant with a yellow (acidic) butt indicates glucose fermentation, but no lysine decarboxylation took place; degradation of peptone accounts for alkalinization of the slant.

If the organism produces lysine deaminase, the resulting deamination reaction will produce compounds that react with the ferric ammonium citrate and produce a red color. Deamination reactions require the presence of oxygen. Therefore, any evidence of deamination will be seen only in the slant. A red slant with yellow (acidic) butt indicates lysine deamination.

Hydrogen sulfide (H_2S) is produced in lysine iron agar by the anaerobic reduction of thiosulfate. Ferric ions in the medium react with the H_2S to form a black precipitate in the butt (Fig. 5.64). Refer to Figure 5.71 and Table 5-26 for the various reactions and symbols used to record them.

Application

LIA is used to differentiate enterics based on their ability to decarboxylate or deaminate lysine and produce hydrogen sulfide (H_2S). LIA also is used in combination with triple sugar iron agar to identify members of *Salmonella* and *Shigella*, because of the fermentation information provided (neither typically ferments lactose or sucrose).

5.71 Lysine Iron Agar Results ◆ Lysine iron agar tubes illustrating from left to right: R/A; K/A, H_2S, (note the small amount of black precipitate near the middle and the gas production from glucose fermentation at the base); uninoculated control; and K/K (obscured by the black precipitate), H_2S.

TABLE 5-26 LIA Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Purple slant/ purple butt	Lysine deaminase negative; lysine decarboxylase positive	K/K
Purple slant/ yellow butt	Lysine deaminase negative; lysine decarboxylase negative; glucose fermentation	K/A
Red slant/ yellow butt	Lysine deaminase positive; lysine decarboxylase negative; glucose fermentation	R/A
Black precipitate	Sulfur reduction	H_2S

In This Exercise

You will be inoculating three LIA slants. Use heavy inocula and tighten the caps before incubating to maintain anaerobic conditions in the butt. Because the decarboxylase/H_2S–positive organisms being differentiated produce results rapidly, incubation time will be 18 to 24 hours. If your labs are scheduled more than 24 hours apart, have someone remove the tubes from the incubator at the appropriate time and refrigerate them until time to record the results. When entering your data, be sure to write the slant information first, followed by the butt and hydrogen sulfide results (example: K/K, H_2S). Refer to Table 5-26 and Figure 5.71 when making your interpretations.

✓ Materials

Per Student Group

- ☐ Four LIA slants
- ☐ Microbial incinerator and wire inoculating needle, or sterile wooden sticks, or disposable inoculating needle when using BSL-2 organisms
- ☐ Fresh cultures on solid media:
  - *Escherichia coli*
  - *Proteus mirabilis* (BSL-2)
  - *Salmonella typhimurium* (BSL-2)

Medium Recipe

Lysine Iron Agar

• Peptone	5.0 g
• Yeast extract	3.0 g
• Dextrose (glucose)	1.0 g
• L-Lysine hydrochloride	10.0 g
• Ferric ammonium citrate	0.5 g
• Sodium thiosulfate	0.04 g
• Bromocresol purple	0.02 g
• Agar	15.0 g

pH 6.5–6.9 at 25°C

Procedure

Lab One

1. Obtain four LIA slants. Label three slants with the names of the organisms, your name, and the date. Label the fourth slant "control."
2. **Using BSL-2 precautions,** heavily inoculate three slants with the test organisms. Stab the agar butt twice and then streak the slant. Do not inoculate the control. Be sure to properly dispose of the wooden stick/inoculating needle (if used), according to your lab's practices.
3. Incubate all slants with the caps tightened at 35 ± 2°C for 18 to 24 hours.

Lab Two

1. Examine the tubes for characteristic color changes.
2. Record your results in the table provided on the data sheet, page 409.

References

Delost, Maria Dannessa. Page 194 in *Introduction to Diagnostic Microbiology.* St. Louis, MO: Mosby, 1997.

Tille, Patricia M. Page 317 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power, eds. *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media.* Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-22

Lysine Iron Agar

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 5-26, page 407, when recording and interpreting your results.

Organism	Color Result	Symbol	Interpretation (include enzyme)
Control			

QUESTIONS

1 *Consider the uninoculated LIA tube.*

a. *Is it a positive or a negative control?*

b. *What information does it provide?*

2 *LIA is a combination medium. Which test(s) does it replace?*

3 *Lysine iron agar is not designed to identify the ability of organisms to ferment. Why, then, is glucose added to the medium (beyond being just another nutrient)?*

4 *LIA is a complex medium with many ingredients. What would be the consequences of the following mistakes in preparing this medium? Consider each independently.*

a. *Inclusion of 1% dextrose rather than the amount called for in the recipe.*

b. *Omission of L-Lysine hydrochloride.*

c. *Omission of ferric ammonium citrate.*

d. *Omission of sodium thiosulfate.*

e. *Omission of bromocresol purple.*

EXERCISE
5-23

Litmus Milk Medium

Theory

Litmus milk is an undefined medium consisting of skim milk and the pH indicator azolitmin. Skim milk provides nutrients for growth, including lactose for fermentation, and protein in the form of casein. Azolitmin (litmus) is pink at pH 4.5 and blue at pH 8.3. Between these extremes it is light purple.

Four basic reactions occur in litmus milk: lactose fermentation, reduction of litmus, casein coagulation, and casein hydrolysis. In combination these reactions yield a variety of results, each of which can be used to differentiate bacteria. Several possible combinations are described in Table 5-27.

Lactose fermentation acidifies the medium and turns the litmus pink (Fig. 5.72, second tube from the right). This acid reaction typically begins with the splitting of the disaccharide into the monosaccharides glucose and galactose by the enzyme β-galactosidase (Figs. 5.73 and A.1). Accumulating acid may cause the casein to precipitate and form an acid clot (Figs. 5.74 and 5.75). Acid clots solidify the medium and can appear pink or white with a pink band at the top depending on the oxidation-reduction status of litmus.

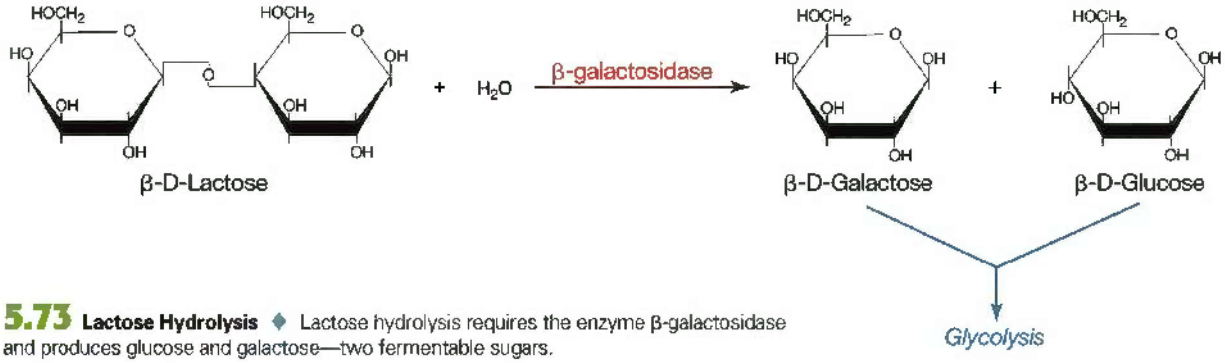
Reduced litmus is white; oxidized litmus is purple. Acid clots can be dissolved in alkaline conditions. Fissures or cracks in the clot are evidence of gas production (Fig. 5.72, third tube from the right at the bottom). Heavy gas production that breaks up the clot is called stormy fermentation.

5.72 Reactions in Litmus Milk ♦ From left to right: alkaline reaction (K); uninoculated control; alkaline reaction (K); digestion reaction (D); acid clot production, gas production, and reduction reaction (ACGR); acid reaction (A); acid reaction, curd production (with whey, the clear liquid, on the surface.) The clear liquid on the surfaces of the D and ACGR tubes is mineral oil to promote anaerobic growth.

TABLE 5-27 Litmus Milk Results and Interpretations*

Table of Results		
Result	**Interpretation**	**Symbol**
Pink color	Acid reaction	A
Pink and solid (white in the lower portion if the litmus is reduced); clot not movable	Acid clot	AC
Fissures in clot	Gas	G
Clot broken apart	Stormy fermentation	S
White color (lower portion of medium)	Reduction of litmus	R
Semisolid and not pink; clear to gray fluid at top	Curd	C
Clarification of medium; loss of "body"	Digestion	D
Blue medium or blue band at top	Alkaline reaction	K
No change	None of the above reactions	NC

* These results may appear together in a variety of combinations

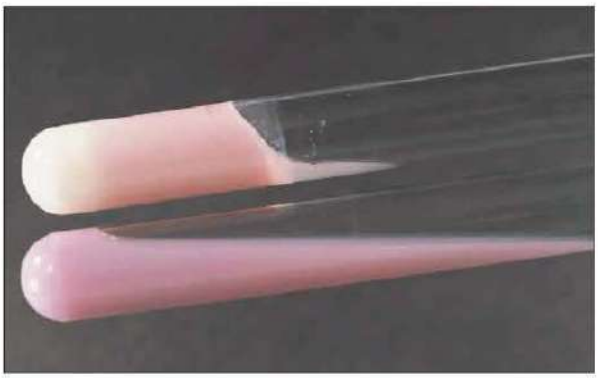


5.73 Lactose Hydrolysis ♦ Lactose hydrolysis requires the enzyme β-galactosidase and produces glucose and galactose—two fermentable sugars.

calcium caseinate (soluble salt of casein) —casease, low pH→ caseinogen (acid clot) (insoluble precipitate)

5.74 Acid Clot Reaction ♦ An acid clot is the result of casease catalyzing the formation of caseinogen, an insoluble precipitate, under acidic conditions.

5



5.75 Acid Clot ♦ An acid clot appears in the top tube; an uninoculated control is below. Note the reduced litmus (white) at the bottom of the clot.

In addition to being a pH indicator, litmus is an E_h (oxidation-reduction) indicator. As mentioned above, reduced litmus is white. If litmus is reduced during lactose fermentation, it will turn the medium white in the lower portion of the tube where the reduction rate is greatest.

Some bacteria produce proteolytic enzymes such as rennin, pepsin, or chymotrypsin that coagulate casein and produce a curd (Figs. 5.72, tube on the far right, and 5.76). A curd differs from an acid clot in that it will not dissolve in alkaline conditions and tends to retract from the sides of the tube, revealing a straw colored fluid called whey.

Certain enzymes can digest both acid clots and curds. A digestion reaction leaves only a clear to brownish fluid behind (Fig. 5.72, center tube). Bacteria that are able only to partially digest the casein typically produce ammonia (NH_3), which raises the pH of the medium and turns the litmus blue. Formation of a blue or purple ring at the top of the clear fluid or bluing of the entire medium indicates an alkaline reaction (Fig. 5.72, tubes on far left and third from left).

Application

Litmus milk is used primarily to differentiate members within the genus *Clostridium*. It differentiates *Enterobacteriaceae* from other Gram negative bacilli based on the ability of enterics to reduce litmus. Litmus milk also is used to cultivate and maintain cultures of lactic acid bacteria.

In This Exercise

You will observe several different reactions in litmus milk. Most tubes will illustrate more than one reaction, so refer to Figure 5.72 and Table 5.27 when making your interpretations.

✓ Materials

Per Student Group

- ☐ Six litmus milk tubes
- ☐ Sterile mineral oil
- ☐ Fresh cultures of:
  - *Alcaligenes faecalis*
  - *Clostridium sporogenes*
  - *Clostridium acetobutylicum*
  - *Escherichia coli*
  - *Lactococcus lactis*

casein (soluble) —rennin, Ca^{++}→ paracasein (soft curd) (insoluble precipitate)

5.76 Curd Formation Reaction ♦ Rennin is only one of several enzymes that curdle milk. It converts casein to paracasein to form the curd.

Medium Recipe

- Litmus milk medium
- Skim milk 100.0 g
- Azolitmin 0.5 g
- Sodium sulfite 0.5 g
- Distilled or deionized water 1.0 L

pH 6.3–6.7 at 25°C

Procedure

Lab One

1. Obtain six litmus milk tubes. Label five tubes with the names of the organisms, your name, and the date. Label the sixth tube "control."
2. Inoculate five tubes with the test cultures. Do not inoculate the control.
3. With a sterile transfer pipette, layer about 1 cm of sterile mineral oil on the surface of the two *Clostridium* cultures.
4. Incubate all tubes aerobically at 35 ± 2°C for 7 to 14 days.

Lab Two

1. Examine the tubes for color changes, gas production, and clot formation. Be sure to compare all tubes to the control, and refer to Table 5-27 when making your interpretations.
2. Record your results in the table provided on the data sheet, page 415.

References

MacFaddin, Jean F. Page 294 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Tille, Patricia M. Page 212 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Zimbro, Mary Jo and David A. Power, eds. Page 309 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-23

Litmus Milk Medium

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 5-27, page 411, when recording and interpreting your results below.

Organism	Color Result	Symbol	Interpretation (include enzyme)
Control			

QUESTIONS

1 *Consider an acid clot. It can appear as pink or white with a pink band at the top.*

a. *Explain the different conditions that would produce each of these occurrences.*

b. *If the reaction to produce a white color occurs, why does the surface remain pink?*

2 *What do you think is the principal difference between a bacterial species that produces a curd in litmus milk and a species that does not?*

5

3 *What reaction would you predict from an organism growing in litmus milk that has the following results in other media? (If you haven't done a particular exercise, omit the question.)*

a. *a clear zone around the growth on a milk agar plate (Exercise 5-16)*

b. *A/– in PR lactose broth (Exercise 5-3)*

c. *A/G in PR lactose broth (Exercise 5-3)*

d. *K in PR glucose broth*

Antimicrobial Susceptibility Testing

Antimicrobials are substances used to control or kill microorganisms. Some are produced naturally by microorganisms; others are synthetics produced in the laboratory. Most bacteria show susceptibility to one or more antimicrobial agents and, as illustrated in Exercise 7-3, may even react in predictable ways to the agent.

Susceptibility testing is frequently used in medicine to identify the antibiotic needed to treat an infection. But because of the predictable responses of some organisms to specific agents, susceptibility testing can also be helpful in microbial identification. All of the antibacterial susceptibility tests included in this unit apply the **disk diffusion method** and are used to aid in bacterial identification.

In the disk diffusion method, a commercially prepared filter paper disk impregnated with a specific concentration of antibiotic is placed on an agar plate immediately after it is inoculated to produce a "bacterial lawn." During incubation, two things happen simultaneously: (1) the antibacterial agent diffuses from the disk into the agar, becoming less concentrated the farther it travels; and (2) bacterial growth slowly develops on the agar surface.

In the area where antibiotic concentration in the agar is sufficient, growth of susceptible bacteria will be inhibited, which results in a clearing surrounding the disk called the **zone of inhibition.** The size of the zone relates to the **minimum inhibitory concentration (MIC)** of the antibacterial agent that is effective against the test organism.

The Clinical Laboratory Standards Institute (CLSI) periodically publishes a Performance Standards for Antimicrobial Testing, which includes Zone Diameter Interpretive Charts for use in testing and determining bacterial susceptibility or resistance to various antibacterial agents. The charts list the concentrations of all currently used antibacterial agents and their applicable zone diameters.

It is important to understand that even resistant organisms may be inhibited by heavy concentrations of an antimicrobial agent. Therefore, the *size* of the zone, not the mere presence of a zone, is what determines susceptibility or resistance. Additionally, specificity of disk diffusion tests is maintained largely by the amount of antibacterial agent added to the disk. For example, many streptococci are susceptible to heavy concentrations of optochin, but resistant to small concentrations. Conversely, *S. pneumoniae* is susceptible to very small concentrations of optochin. By preparing disks with a scant 5 µg of optochin, the test system is able to accurately differentiate *S. pneumoniae* from other organisms in the genus. ◆

Bacitracin, Novobiocin, and Optochin Susceptibility Tests

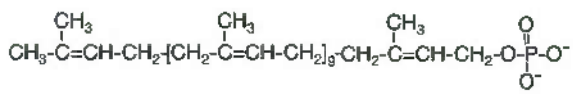
EXERCISE 5-24

Theory

Bacitracin, produced by *Bacillus licheniformis*, is a powerful peptide antibiotic that inhibits bacterial cell wall synthesis by interfering with transport of peptidoglycan subunits across the cytoplasmic membrane (Fig. 5.77). Thus, it is effective only on bacteria that have cell walls and are in the process of growing. In this test, any zone of clearing 10 mm or greater around the disk is interpreted as bacitracin susceptibility (Fig. 5.78). For more information on antimicrobial susceptibility, refer to Exercise 7-3, Antimicrobial Susceptibility Test.

Novobiocin is an antibiotic, produced by *Streptomyces niveus*. It is related to coumarin and interferes with ATPase activity associated with DNA gyrase, an enzyme necessary during DNA replication. In this test, a 5 µg disk should produce a zone of clearing 16 mm or more for the organism to be considered novobiocin susceptible (Fig. 5.79).

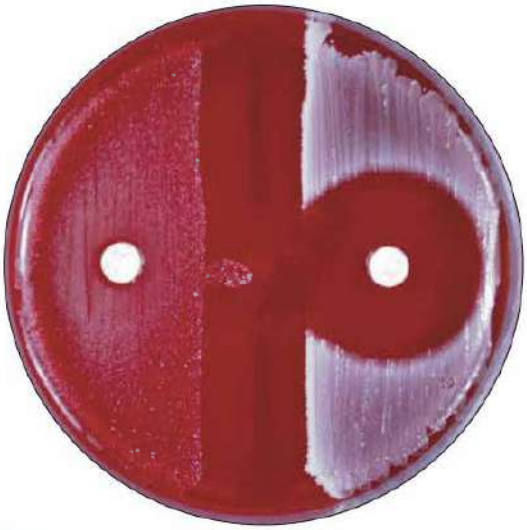
Optochin is an antibiotic derived from quinine that interferes with ATP synthase activity and ATP production in susceptible bacteria. A 6 mm disk (containing 5µg of



5.77 Undecaprenyl Phosphate ◆ Undecaprenyl phosphate, a C_{55} molecule derived from 11 isoprene subunits plus a phosphate, is involved in transporting peptidoglycan subunits across the cell membrane during cell wall synthesis. Bacitracin interferes with its release from the peptidoglycan subunit.

optochin) should produce a zone of inhibition 14 mm or more for the organism to be considered optochin susceptible (Fig. 5.80).

5.78 Bacitracin Susceptibility Test on a Sheep Blood Agar Plate ◆ The organism above has no clear zone and is resistant (R); the organism below has a clear zone larger than 10 mm and is susceptible (S). (The disks themselves are usually 6 mm.)



5.79 Novobiocin Susceptibility Test on a Sheep Blood Agar Plate ◆ A novobiocin-resistant organism (R) is on the left; a susceptible organism (S) is on the right.

5.80 Optochin Susceptibility Test on a Sheep Blood Agar Plate ◆ The zone of inhibition surrounding the disk indicates susceptibility to optochin and presumptive identification of *S. pneumoniae*.

Application

The bacitracin test is used to differentiate and presumptively identify β-hemolytic group A streptococci (*Streptococcus pyogenes*–bacitracin susceptible) from other β-hemolytic streptococci (bacitracin resistant). It also differentiates the genus *Staphylococcus* (resistant) from the susceptible *Micrococcus*.

The novobiocin test is used to differentiate coagulase-negative staphylococci. Most frequently it is used to presumptively identify the novobiocin-resistant *Staphylococcus saprophyticus*.

The optochin test is used to presumptively differentiate *Streptococcus pneumoniae* from other α-hemolytic streptococci.

In This Exercise

You will inoculate from one to three blood agar plates (depending on the tests chosen by your instructor) to produce confluent growth of the test organisms. You will then place specific antibiotic disks in the center of the inoculum. Following incubation, you will measure the clear zones surrounding the disks to determine susceptibility or resistance of the test organisms to the antibiotics.

✓ Materials

Per Student Group

- ☐ One blood agar plate per test (commercial preparation of TSA containing 5% sheep blood)
- ☐ Sterile cotton applicators
- ☐ Screw cap jar of alcohol with forceps

Bacitracin Test

- ☐ 0.04 unit bacitracin disks (A disks)
- ☐ Fresh broth cultures of:
  - *Staphylococcus aureus* (BSL-2)
  - *Micrococcus luteus*

Novobiocin Test

- ☐ 5 µg novobiocin disks
- ☐ Fresh broth cultures of:
  - *Staphylococcus epidermidis*
  - *Staphylococcus saprophyticus*

Optochin Test

- ☐ 5 µg (6 mm) optochin disks (P disks)
- ☐ Fresh broth cultures of:
  - *Streptococcus pneumoniae* (BSL-2)
  - *Streptococcus agalactiae* (BSL-2)

Procedure (Per Test)

Lab One

1. **Use BSL-2 precautions as appropriate** for the tests and organisms your class is assigned.
2. Obtain one blood agar plate for each test you are performing, and draw a line on the bottom dividing the plate into two halves. Using a sterile cotton applicator, inoculate half of the plate with one of the two test organisms. (Make the inoculum as light as possible by wiping and twisting the wet cotton swab on the inside of the culture tube before removing it.) Inoculate the plate by making a single streak nearly halfway across its diameter. Turn the plate 90° and spread the organism evenly to produce a bacterial lawn covering nearly half the agar surface. Refer to the photo in Figure 5.78.
3. Being careful not to mix the cultures, repeat the process on the other half of the plate using the second test organism. Allow the broth to be absorbed by the agar for 5 minutes before proceeding to step 4.
4. Alcohol-flame forceps by placing them in the Bunsen burner flame long enough to ignite the alcohol. (*Note*: Do not hold the forceps in the flame and do not hold them near the alcohol jar. If the alcohol jar should ignite, smother the flame with the jar's lid.) Once the alcohol has burned off, use the forceps to place an antibacterial disk in the center of the half containing the first organism. Gently tap the disk into place to ensure that it makes full contact with the agar surface. Return the forceps to the alcohol.
5. Repeat step 4, placing a disk on the other half of the plate—the area containing the second organism. Tap the disk into place and return the forceps to the alcohol.
6. Invert the plate, label it appropriately, and incubate it at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Remove the plate from the incubator and examine it for clearing around the disks. Refer to Tables 5-28, 5-29, and 5-30 to interpret your results.
2. Record your results in the table on the data sheet, page 421.

TABLE **5-28** **Bacitracin Test Results and Interpretations**

Table of Results		
Result	**Interpretation**	**Symbol**
Zone of clearing 10 mm or greater	Organism is susceptible to bacitracin	S
Zone of clearing less than 10 mm	Organism is resistant to bacitracin	R

TABLE 5-29 Novobiocin Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Zone of clearing 16 mm or greater	Organism is susceptible to novobiocin	S
Zone of clearing less than 16 mm	Organism is resistant to novobiocin	R

TABLE 5-30 Optochin Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Zone of clearing 14 mm or greater	Organism is susceptible to optochin	S
Zone of clearing less than 14 mm	Organism is resistant to optochin	R

5

References

Delost, Maria Dannessa. Page 107 in *Introduction to Diagnostic Microbiology.* St. Louis, MO: Mosby, 1997.

DIFCO Laboratories. Page 292 in *DIFCO Manual,* 10th ed. Detroit: DIFCO Laboratories, 1984.

MacFaddin, Jean F. Page 3 in *Biochemical Tests for Identification of Medical Bacteria,* 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Tille, Patricia M. Page 198 and 222 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckenberger, and Gail Woods. Pages 644–645, 1471–1472, and 1474 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology,* 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-24

Bacitracin, Novobiocin, and Optochin Susceptibility Tests

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Tables 5-28, 5-29, and 5-30, pages 419 and 420, when recording and interpreting your results in the table below.

Antibacterial Agent	Organism	Zone Diameter (mm)	Susceptible (S) or Resistant (R)
Bacitracin			
Novobiocin			
Optochin			

QUESTIONS

1 *Why should these tests be run with positive and negative controls?*

2 *Why is it important to get a bacterial lawn rather than isolated colonies on the plate?*

5

3 *Does the zone of inhibition's edge indicate the limit of antibacterial agent diffusion into the agar? Give reasons or evidence to support your answer.*

Other Differential Tests

This set of exercises includes tests that do not fit elsewhere but are important to consider. Blood agar is especially useful for detecting hemolytic ability of Gram-positive cocci—typically *Streptococcus* species. It also is used as a general-purpose growth medium appropriate for fastidious and nonfastidious microorganisms alike. Also targeting *Streptococcus* species is the CAMP test. The coagulase and clumping factor tests are commonly used to presumptively identify pathogenic *Staphylococcus aureus*. Motility agar is used to detect bacterial motility, especially in differentiating *Enterobacteriaceae* and other Gram-negative rods. ◆

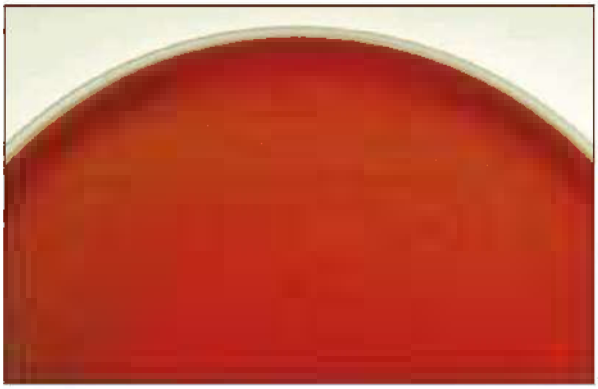
EXERCISE
5-25
Blood Agar

5

Theory

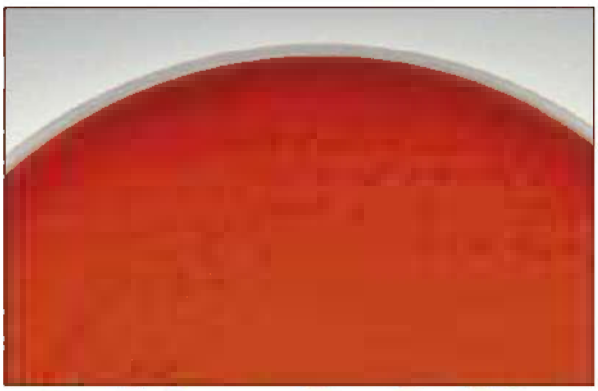
Several species of Gram-positive cocci produce exotoxins called **hemolysins**, which are able to destroy red blood cells (RBCs) and hemoglobin. Blood agar includes 5% blood (frequently sheep blood) in a tryptic soy agar base. It allows differentiation of bacteria based on their ability to hemolyze RBCs.

The three major types of hemolysis are β-hemolysis, α-hemolysis, and γ-hemolysis. β-hemolysis, the complete destruction of RBCs and hemoglobin, results in a clearing of the medium around the colonies (Fig. 5.81). α-hemolysis is the partial destruction of RBCs and produces a greenish discoloration of the agar around the colonies (Fig. 5.82). Finally, γ-hemolysis is actually nonhemolysis and appears as simple growth with no change to the medium (Fig. 5.83).



5.82 α-Hemolysis ◆ This is a streak plate of *Streptococcus pneumoniae* demonstrating α-hemolysis. The greenish zone around the colonies results from incomplete lysis of red blood cells. This photograph was taken with transmitted light.

5.81 β-Hemolysis ◆ *Streptococcus pyogenes* demonstrates β-hemolysis. The clearing around the growth is a result of complete lysis of red blood cells. This photograph was taken with transmitted light.



5.83 γ-Hemolysis ◆ This streak plate of *Staphylococcus epidermidis* on a sheep blood agar illustrates no hemolysis. This photograph was taken with transmitted light.

Hemolysins produced by streptococci are called **streptolysins**. They come in two forms—type O and type S. **Streptolysin O** is oxygen labile and expresses maximal activity under anaerobic conditions. **Streptolysin S** is oxygen stable but expresses itself optimally under anaerobic conditions as well. The easiest method of providing an environment favorable for streptolysins on blood agar is what is called the **streak-stab technique.** In this procedure the blood agar plate is streaked for isolation and then stabbed with a loop. The stabs encourage streptolysin activity because of the reduced oxygen concentration of the subsurface environment (Fig. 5.84).

Application

Blood agar is used for isolation and cultivation of many types of fastidious bacteria. It also is used to differentiate bacteria based on their hemolytic characteristics, especially within the genera *Streptococcus*, *Enterococcus*, and *Aerococcus*.

5

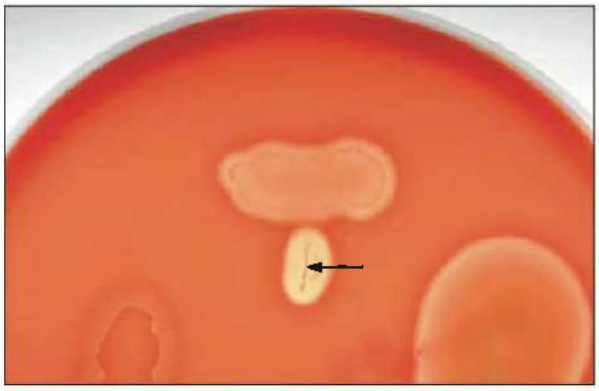
In This Exercise

You will not be provided with organisms to inoculate your plate. Instead, your partner will take a swab of your throat. Then you will inoculate the blood agar plate in one quadrant to begin a streak for isolation. Isolation of the different colonies is the only way to observe the different forms of hemolysis properly. (*Note*: When you streak your plate, do only the first streak with the swab, and do streaks two, three, and four with your inoculating loop. If you are not sure how to do this, refer to Exercise 1-4. Because of the potential for cultivating a pathogen, tape down the plate lid immediately after streaking. Upon removing your plate from the incubator, do not open it unless given permission by your instructor.)

✓ Materials

Per Student Group

- ☐ One sheep blood agar plate (commercially available—TSA containing 5% sheep blood)
- ☐ Sterile cotton swabs
- ☐ Sterile tongue depressors
- ☐ Disposable loops



5.84 Aerobic Versus Anaerobic Hemolysis ◆ An unidentified throat culture isolate demonstrates α-hemolysis when growing on the surface, but β-hemolysis beneath the surface surrounding the stab (arrow). This results from production of an oxygen-labile hemolysin.

Medium Recipe

5% Sheep Blood Agar (TSA ~ 5% Sheep Blood)

• Infusion from beef heart (solids)	2.0 g
• Pancreatic digest of casein	13.0 g
• Sodium chloride	5.0 g
• Yeast extract	5.0 g
• Agar	15.0 g
• Defibrinated sheep blood	50.0 mL
• Distilled or deionized water	1.0 L
pH 7.1–7.5 at 25°C	

Procedure

Lab One

1. Have your lab partner obtain a culture from your throat. Follow the procedure in Appendix B.
2. Immediately transfer the specimen to a blood agar plate. Use the swab to begin a streak for isolation. Refer to Exercise 1-4, page 43 if necessary.
3. Dispose of the swab in a container designated for autoclaving.
4. Finish the isolation procedure by streaking quadrants 2, 3, and 4 with a different plastic disposable loop for each. Immediately dispose of each loop properly when done except for the last one (see step 5).
5. After completing the last streak, use your loop to stab the agar in two or three places in the first streak pattern, and then in two or three places not previously inoculated. Properly dispose of the loop.

6. Label the plate with your name, the specimen source ("throat culture"), and the date.
7. Tape down the lid to prevent it from opening accidentally (Fig. I.5). Invert and incubate the plate aerobically at 35 ± 2°C for 24 hours. Remove the plate to the refrigerator if you cannot examine it at 24 hours.

Lab Two

1. After incubation, do not open your plate until your instructor has seen it and given permission to do so.
2. Using transmitted and reflected light as necessary, and referring to Table 5-31, observe for color changes and clearing around the colonies. This can be done using a colony counter or by holding the plate up to a light. Record your results in the table on the data sheet, page 427.

TABLE 5-31 Blood Agar Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Clearing around growth	Organism hemolyzes RBCs completely	β-hemolysis
Greening around growth	Organism hemolyzes RBCs partially	α-hemolysis
No change in the medium	Organism does not hemolyze RBCs	no (γ) hemolysis

References

Delost, Maria Dannessa. Page 103 in *Introduction to Diagnostic Microbiology.* St. Louis, MO: Mosby, 1997.

Krieg, Noel R. Page 619 in *Methods for General and Molecular Bacteriology.* Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Power, David A. and Peggy J. McCuen. Page 115 in *Manual of BBL™ Products and Laboratory Procedures,* 6th ed. Cockeysville, MD: Becton Dickinson Microbiology Systems, 1988.

Tille, Patricia M. Page 64 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckenberger, and Gail Woods. Chap.13 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology,* 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Zimbro, Mary Jo and David A. Power, eds. *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media.* Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-25

Blood Agar

OBSERVATIONS AND INTERPRETATIONS

1 Choose four different colonies (with a diversity of hemolysis reactions, including one stab) and fill in the table. Refer to Table 5-31, page 425, when recording and interpreting your results.

Source of Culture	Colony Morphology	Hemolysis Result	Interpretation

QUESTIONS

1 *The streak-stab technique, used to promote streptolysin activity, is preferred over incubating the plates anaerobically.*

a. *Why do you think this is so?*

b. *Compare and contrast what you see as the advantages and disadvantages of each procedure.*

2 *Assuming that all of the organisms cultivated in this exercise came from the throats of healthy students, why is it important to cover and tape the plates?*

5

3 *Why is the streak plate preferred over the spot inoculations in this procedure?*

EXERCISE
5-26

CAMP Test

Theory

Group B *Streptococcus agalactiae* produces the CAMP factor—a hemolytic protein that acts synergistically with the β-hemolysin of *Staphylococcus aureus* subsp. *aureus*. When streaked perpendicularly to an *S. aureus* subsp. *aureus* streak on blood agar (Fig. 5.85), an arrowhead-shaped zone of hemolysis forms and is a positive result.

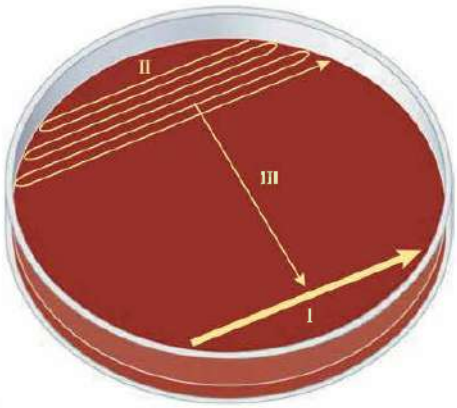
Application

The CAMP test (an acronym of the developers of the test—Christie, Atkins, and Munch-Peterson) is used to differentiate Group B *Streptococcus agalactiae* (+) from other *Streptococcus* species (−).

In This Exercise

You will inoculate a blood agar plate as shown in Figure 5.86 with *Streptococcus agalactiae* and *Staphylococcus aureus*. Following incubation, you will examine your plate for the characteristic arrowhead pattern of clearing.

5.85 Positive CAMP Test Results ◆ Note the arrowhead zone of clearing in the region where the CAMP factor of *Streptococcus agalactiae* acts synergistically with the β-hemolysin of *Staphylococcus aureus* subsp. *aureus*.



5.86 CAMP Test Inoculation ◆ Two inoculations are made. First *Staphylococcus aureus* subsp. *aureus* is streaked along one edge of a fresh blood agar plate (I). Then the isolate (when testing an unknown organism) is inoculated densely in the other half of the plate opposite *S. aureus* (II). Finally, a single streak is made from inside streak II toward, but not touching, *S. aureus* (III).

Materials

- ☐ Two blood agar plates (commercial preparation of TSA containing 5% sheep blood)
- ☐ Sterile cotton applicators
- ☐ Fresh broth cultures of:
  - *Staphylococcus aureus* subsp. *aureus* (BSL-2)
  - *Streptococcus agalactiae* (BSL-2)
  - *Streptococcus pneumoniae* (BSL-2)

Procedure

Lab One

1. **Use BSL-2 precautions in inoculating the plates.**
2. Obtain two blood agar plates
3. Using a cotton swab, inoculate both plates as shown in Figure 5.86, each with a single streak of *S. aureus* along one edge (streak I).
4. Inoculate each plate with a dense smear of one of the other two organisms across from the *S. aureus* streak, as shown in streak II. Finish each plate with a single streak (as in streak III) from the *Streptococcus* species toward, but not touching, the *S. aureus*. ***Note:*** It works best and saves material to do this third streak

with the same swab used in streak II before moving on to the other plate. Repeat procedure in the second plate with *S. aureus* and *S. pneumoniae*.

5 Label the plates with your name, the date, and the names of the organisms. Tape them closed as in Figure I.5.

6 Invert the plates and incubate them at 35 ± 2°C for 24 hours.

Lab Two

1 Do not open the plates.

2 Remove the plates from the incubator and observe for the characteristic arrowhead pattern.

3 Record your results in the table on the data sheet, page 431, referring to Table 5-32 to interpret your results.

4 Discard the plates in an appropriate biohazard or autoclave container.

5

TABLE 5-32 CAMP Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Arrowhead pattern	Organism produces hemolytic CAMP protein. Presumptively identified as *S. agalactiae*.	+
No arrowhead pattern	Organism does not produce hemolytic CAMP protein. Presumptively identified as *not S. agalactiae*.	–

References

Murray, Patrick R., Ken S. Rosenthal, and Michael A. Pfaller. Page 250 in *Medical Microbiology*, 5th ed. Philadelphia: Elsevier Mosby, 2005.

Spellerberg, Barbara and Claudia Brandt. Pages 239–240 in *Manual of Clinical Microbiology*, 10th ed. James Versalovic, et al., eds. Washington, DC: ASM Press, 2011.

Tille, Patricia M. Pages 200 and 257 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckenberger, and Gail Woods. Pages 717 and 1468 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-26

CAMP Test

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 5-32, page 430, when recording and interpreting your results in the table below.

Organism (interacting with *S. areus*)	**Result** + / −	**Interpretation**

QUESTIONS

1 *You were instructed to not allow streak I and streak III to touch. Why do you think this is important?*

2 *Consider the arrowhead region of clearing.*

a. *Why do you think the clearing in a positive CAMP test is an arrowhead shape and not some other shape?*

b. *Would it likely make an important difference if you began streak II near* S. aureus *and streaked across the plate in the other direction? Why?*

3 *What result would you expect to get if you accidentally reverse the organisms in the procedure?*

Coagulase and Clumping Factor Tests

EXERCISE 5-27

Theory

Staphylococcus aureus is an opportunistic pathogen that can be highly resistant to both the normal immune response and antimicrobial agents. Its resistance is due, in part, to the production of a coagulase enzyme. Plasma is the fluid portion of blood and includes, among other components, clotting factors. Coagulase works in conjunction with normal plasma components to form protective fibrin barriers around individual bacterial cells or groups of cells, shielding them from phagocytosis and other types of attack.

Coagulase enzymes occur in two forms—**free coagulase** and **bound coagulase**. Free coagulase is an extracellular enzyme (released from the cell) that reacts with a plasma component called coagulase-reacting factor (CRF). The resulting reaction is similar to the conversion of prothrombin and fibrinogen in the normal clotting mechanism. Bound coagulase, also called **clumping factor**, is attached to the bacterial cell wall and reacts directly with fibrinogen in plasma. The fibrinogen then precipitates, causing the cells to clump together in a visible mass.

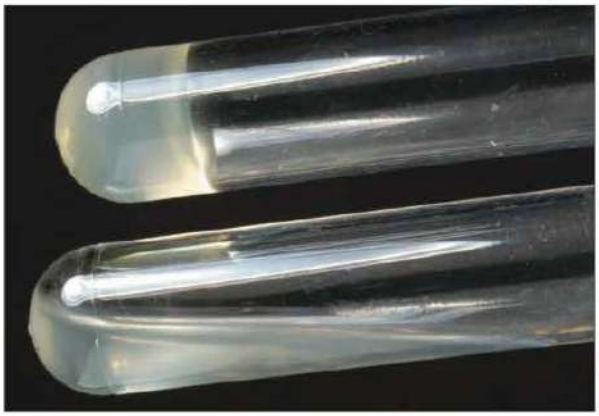
Two forms of the coagulase test have been devised to detect the enzymes: the tube test and the slide test. The tube test detects the presence of either bound or free coagulase, and the slide test detects only bound coagulase. Both tests utilize rabbit plasma treated with anticoagulant to interrupt the normal clotting mechanisms.

The tube test is performed by adding the test organism to rabbit plasma in a test tube. Coagulation of the plasma (including any thickening or formation of fibrin threads) within 24 hours indicates a positive reaction (Fig. 5.87). The plasma typically is examined for clotting (without shaking) periodically for about 4 hours. After 4 hours coagulase-negative tubes can be incubated overnight, but no more than a total of 24 hours, because coagulation can take place early and revert to liquid within 24 hours.

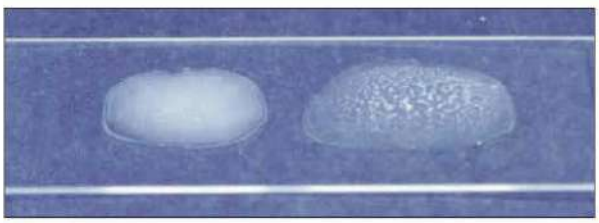
In the slide test, bacteria are transferred to a slide containing a small amount of plasma. Agglutination of the cells on the slide within 1 to 2 minutes indicates the presence of bound coagulase (Fig. 5.88). Equivocal or negative slide test results typically are given the tube test for confirmation.

Application

The coagulase test typically is used to differentiate *Staphylococcus aureus* from other Gram-positive cocci.



5.87 Coagulase Tube Test Results in Rabbit Plasma ♦ These coagulase tubes illustrate a coagulase-negative (−) organism, below, and a coagulase-positive (+) organism, above. The tube test identifies both bound and free coagulase enzymes. Coagulase increases bacterial resistance to phagocytosis and antibodies by surrounding infecting organisms with a clot.



5.88 Coagulase Slide Test (Clumping Factor) ♦ This slide illustrates a coagulase-negative (−) organism on the left and a coagulase-positive (+) organism on the right. Agglutination of the coagulase plasma is indicative of a positive result for bound coagulase. **Use BSL-2 precautions when performing this test** with an unknown organism or *S. aureus*.

In This Exercise

You will perform both tube and slide coagulase tests. Immediately enter your slide test results on the data sheet, page 435. Read the tube test the following day.

✓ Materials

Per Student Group

- ☐ Four sterile rabbit plasma tubes (0.5 mL in 12 mm × 75 mm test tubes)
- ☐ Sterile 1 mL transfer pipettes
- ☐ Six sterile wooden sticks or disposable plastic loops

- ☐ Sterile saline (0.9% NaCl)
- ☐ Two microscope slides
- ☐ Fresh slant cultures of:
  - *Staphylococcus aureus* (BSL-2)
  - *Staphylococcus epidermidis*

Procedure

***Note*: Use BSL-2 precautions for both tests.**

Lab One: Tube Test

1. Obtain three coagulase tubes. Label two tubes with the names of the organisms, your name, and the date. Label the third tube "control."
2. Using a sterile wooden stick or disposable loop, inoculate two tubes with the test organisms. Immediately dispose of the stick/loop properly. Then, mix the contents by gently rolling the tube between your hands. Do not inoculate the control.
3. Incubate all tubes at 35 ± 2°C for up to 24 hours, checking for coagulation periodically for the first 4 hours.

Lab One: Slide Test (Clumping Factor)

1. Obtain two microscope slides, and divide them in half with a marking pen. Label the sides A and B.
2. Place a drop of sterile saline on side A and a drop of coagulase plasma (from the fourth coagulase tube) on side B of each slide.
3. Using a different sterile wooden stick or a disposable loop for each, transfer a loopful of *S. aureus* to each half of one slide, making sure to completely (but gently) emulsify the bacteria in the solutions. Immediately dispose of the stick/loop properly. Gently rock the slide for 5 to 10 seconds, and then observe for agglutination within 2 minutes. Clumping after 2 minutes is not a positive result.
4. Repeat step 3 using the other slide and *S. epidermidis*.
5. Record your results in the table on the data sheet. Refer to Figure 5.88 and Table 5-33 when making your interpretations. Confirm any negative results by comparing with the completed tube test in 24 hours.

TABLE 5-33 Coagulase Slide Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Clumping of cells	Plasma has been coagulated	+
No clumping of cells	Plasma has not been coagulated	−

Lab Two

1. Remove all tubes from the incubator no later than 24 hours after inoculation. Examine for clotting of the plasma.
2. Record your results on the data sheet. Refer to Figure 5.87 and Table 5-34 when making your interpretations.

TABLE 5-34 Coagulase Tube Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Medium is solid within 24 hours	Plasma has been coagulated	+
Medium remains liquid within 24 hours	Plasma has not been coagulated	−

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 111 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Delost, Maria Dannessa. Pages 98–99 in *Introduction to Diagnostic Microbiology*. St. Louis, MO: Mosby, 1997.

DIFCO Laboratories. Page 232 in *DIFCO Manual*, 10th ed. Detroit: DIFCO Laboratories, 1984.

Holt, John G., ed. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Baltimore: Lippincott Williams and Wilkins, 1994.

Lányi, B. Page 62 in *Methods in Microbiology*, Vol. 19. R. R. Colwell and R. Grigorova, eds. New York: Academic Press, 1987.

MacFaddin, Jean F. Page 105 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Tille, Patricia M. Page 203 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-27

Coagulase and Clumping Factor Tests

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Tables 5-33 and 5-34, page 434, when recording and interpreting your results in the tables below.

Slide Test Results			
Organism	**Slide**	**Result**	**Interpretation** (include coagulase type)
	Side A		
	Side B		
	Side A		
	Side B		

Tube Test Results		
Organism	**Result**	**Interpretation** (include coagulase type)
Uninoculated control		

QUESTIONS

1 *Consider the controls.*

a. *Are the uninoculated tube and the saline on the slide positive or negative controls?*

b. *What information is provided by the uninoculated tube in the tube test?*

c. *What information is provided by the saline solution in the slide test?*

5

d. *Why is it a good idea to run a positive control in both the slide and tube tests?*

2 *Why is it more important to use fresh cultures in the coagulase test than in a test medium such as milk agar or phenol red broth?*

3 *How would you interpret a negative slide test and a positive tube test using the same organism?*

4 *List possible reasons why the slide test is not appropriate for detecting free coagulase.*

EXERCISE
5-28
Motility Test

Theory

Motility test agar is a semisolid medium designed to detect bacterial motility. Its agar concentration is reduced from the typical 1.5% to 0.4%—just enough to maintain its form while allowing movement of motile bacteria. It is inoculated by stabbing with a straight transfer needle. Motility is detectable as diffuse growth radiating from the central stab line.

A tetrazolium salt (TTC) sometimes is added to the medium to make interpretation easier. TTC is used by the bacteria as an electron acceptor. In its oxidized form, TTC is colorless and soluble; when reduced it is red and insoluble (Fig. 5.89). A positive result for motility is indicated when the red (reduced) TTC is seen radiating outward from the central stab. A negative result shows red only along the stab line (Fig. 5.90).

Application

This test is used to detect bacterial motility. Motility is an important differential characteristic of *Enterobacteriaceae* and other groups.

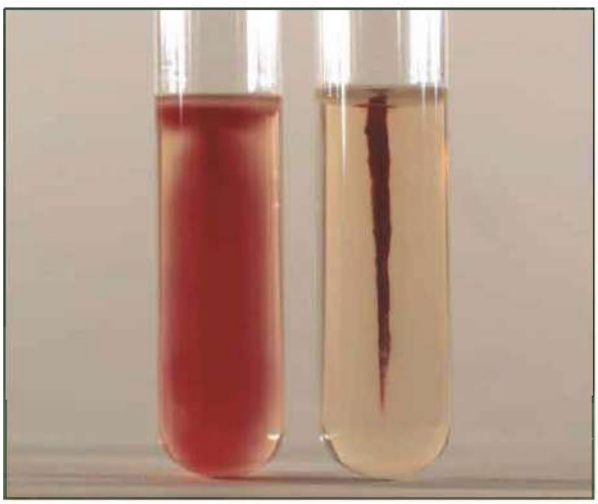
In This Exercise

You will inoculate two motility stabs with an inoculating needle. Straighten the needle before you stab the medium. It is important, also, to stab straight into the medium and remove the needle along the same line. Lateral movement of the needle will make interpretation more difficult.

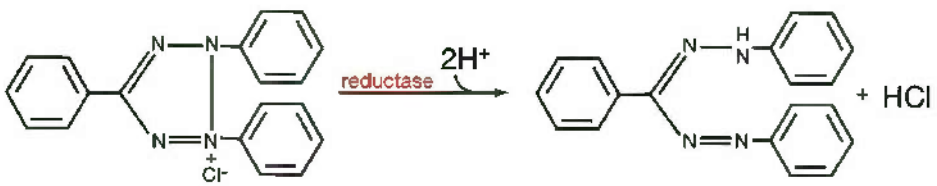
Materials

Per Student Group

- ☐ Three motility test agar tubes
- ☐ Fresh cultures of:
  - *Enterobacter aerogenes*
  - *Streptococcus* spp. or *Staphylococcus* spp.



5.90 Motility Test Results ◆ These motility test media were inoculated with a motile (+) organism on the left and a nonmotile (−) organism on the right.



2,3,5-Triphenyltetrazolium chloride$_{oxidized}$ (TTC_{ox})
colorless and soluble

Formazan$_{reduced}$
red color and insoluble

5.89 Motility Indicator Reaction: Reduction of TTC ◆
Reduction of 2,3,5-Triphenyltetrazolium chloride (TTC) by metabolizing bacteria results in its conversion from colorless and soluble to the red and insoluble compound formazan. The location of growing bacteria can be determined easily by the location of the formazan in the medium.

Medium Recipe

Motility Test Medium

- Beef extract 3.0 g
- Pancreatic digest of gelatin 10.0 g
- Sodium chloride 5.0 g
- Agar 4.0 g
- Triphenyltetrazolium chloride (TTC) 0.05 g
- Distilled or deionized water 1.0 L

pH 7.1–7.4 at 25°C

Procedure

Lab One

1. Obtain three motility stabs. Label two tubes, each with the name of the organism, your name, and the date. Label the third tube "control."
2. Using an inoculating needle, stab inoculate two tubes with the test organisms. (Motility can be obscured by the careless stabbing technique. Try to avoid lateral movement when performing this stab.) Stab the control with a sterile inoculating needle.
3. Incubate the tubes aerobically at 35 ± 2°C for 24 to 48 hours.

Lab Two

1. Examine the growth pattern for characteristic spreading from the stab line. Growth will appear red because of the TTC in the medium. Refer to Table 5-35 when interpreting your results.
2. Record your results in the chart provided on the data sheet, page 439.
3. Dispose of tubes properly when finished.

TABLE 5-35 Motility Test Results and Interpretations

Table of Results		
Result	**Interpretation**	**Symbol**
Red diffuse growth radiating outward from the stab line	The organism is motile	+
Red growth only along the stab line	The organism is nonmotile	–

References

MacFaddin, Jean F. Page 327 in *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Tille, Patricia M. Page 216 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckenberger, and Gail Woods. Pages 227–228 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Zimbro, Mary Jo and David A. Power, eds. Page 374 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-28

Motility Test

OBSERVATIONS AND INTERPRETATIONS

1 Refer to Table 5-35, page 438, when recording and interpreting your results in the table below.

Organism	Result	+/–	Interpretation

QUESTIONS

1 *Consider the tube stabbed with the sterile inoculating needle.*

a. *Is this a positive or a negative control?*

b. *What information is provided by the sterile-stabbed tube?*

2 *Why is it important to carefully insert and remove the needle along the same stab line?*

3 *Consider the TTC indicator.*

a. *Why is it essential that the reduced TTC be insoluble?*

b. *Why is there less concern about the solubility of the oxidized form of TTC?*

Multiple Test Systems

Multiple test systems are systems designed to run an entire battery of tests simultaneously. They employ the same biochemical principles discussed earlier in this section and are read essentially in the same manner. All conditions possible with standard tubed media also can be achieved with multiple test media, including aerobic or anaerobic growth conditions and the addition of reagents for confirmatory testing.

The savings in time and money alone make these systems enormously valuable. Even more important, what might take days or weeks to do with media preparation and individual tests, multiple test systems can do in as little as 18 hours. In addition, these multi-test systems come with databases for fast and easy identification.

These two exercises will give you an opportunity to have fun while using some of the skills you have learned. The organisms chosen for these tests will be provided as unknowns for you to identify. The system you use may be used in conjunction with your bacterial unknown project or with one of the identification exercises in Section 7. ◆

api 20 E Identification System for *Enterobacteriaceae* and Other Gram-negative Rods

EXERCISE 5-29

5

Theory

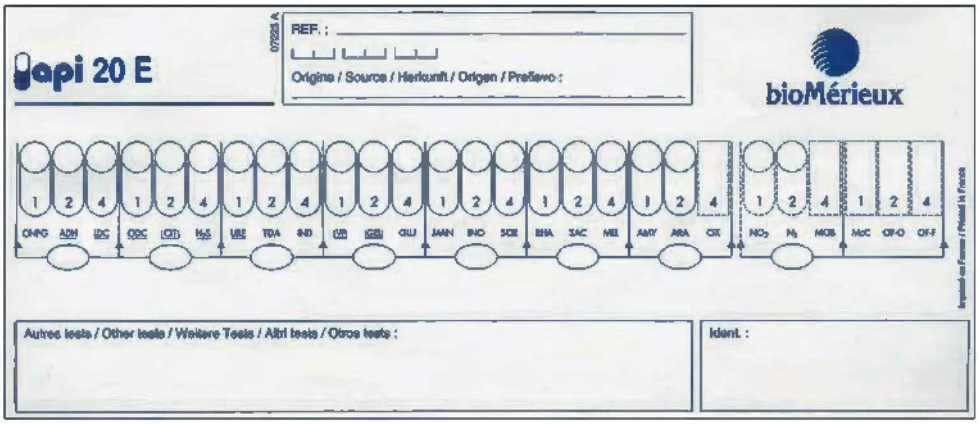
The api 20 E system is a plastic strip of 20 microtubes and cupules, partially filled with different dehydrated substrates. Bacterial suspension is added to the microtubes, rehydrating the media and inoculating them at the same time. As with the other biochemical tests in this section, color changes take place in the tubes either during incubation or after addition of reagents. These color changes reveal the presence or absence of chemical action and, thus, a positive or negative result (Fig. 5.91).

After incubation, spontaneous reactions—those that do not require addition of reagents—are evaluated first. Then tests that require addition of reagents are performed and evaluated. Finally, the results are entered on the api 20 E Result Sheet (Fig. 5.92). An oxidase test is performed separately and constitutes the 21st test.

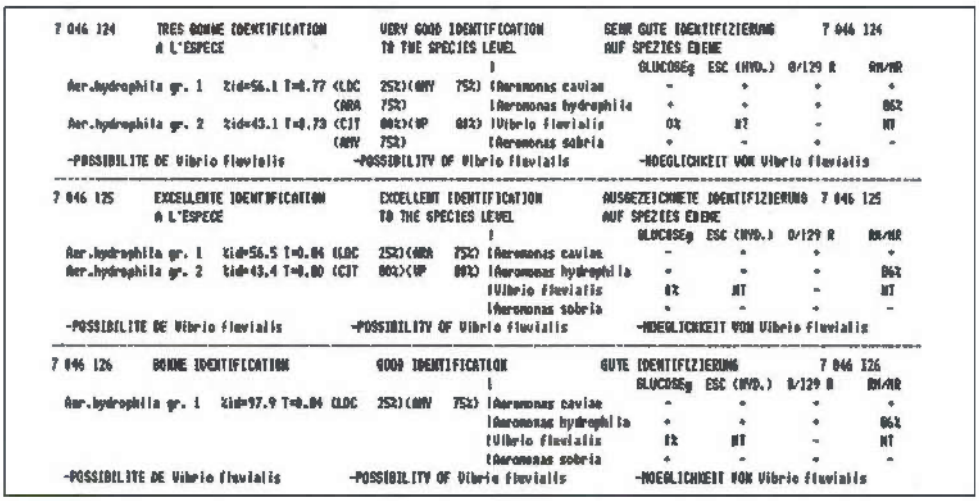
As shown in Figure 5.92, the Result Sheet divides the tests into groups of three, with the members of a group having numerical values of 1, 2, or 4, respectively. These numbers are assigned for positive (+) results only. Negative (–) results are not counted. The values for positive results in each group are added together to produce a number from 0 to 7, which is entered in the oval below the three tests. The totals from each group are combined sequentially to produce a seven-digit code, which can then be interpreted on the Analytical Profile Index[1] (Fig. 5.93).

[1] The index is now available only as a subscription to apiweb through BioMérieux-USA.com: https://apiweb.biomerieux.com

5.91 **api 20 E Test Strips** ◆ The top strip is uninoculated. The bottom strip (with the exception of GEL) illustrates all positive results.


api 20 E
REF. :
Origine / Source / Herkunft / Origen / Proïsevo :
bioMérieux
Autres tests / Other tests / Weitere Tests / Altri tests / Otros tests :
Ident. :

5.92 api 20 E Result Sheet ◆ The api 20 E Result Sheet divides the tests into groups of three, assigning each member of a group a numerical value of 1, 2, or 4. The values for positive results in each group are added together to produce a single-digit number. This number, when combined with the numbers from other groups, produces a seven-digit code that then is interpreted using the Analytical Profile Index available (with subscription) at https://apiweb.biomerieux.com.



5.93 A Portion of the Original Analytical Profile Index (api) ◆ This is an excerpt from the original printed version of the api, but it illustrates the principle behind the identification process. Identification is made by locating the experimentally determined seven-digit number for the isolate.

In rare instances, information from the 21 tests (and the seven-digit code) is not discriminatory enough to identify an organism. When this occurs, the organism is grown and examined on MacConkey agar and supplemental tests are performed for nitrate reduction, oxidation/reduction of glucose, and motility. The results are entered separately in the supplemental spaces on the Result Sheet and used for final identification.

Application

The api 20 E multi-test system (available from BioMérieux, Inc.) is used clinically for the rapid identification of *Enterobacteriaceae* (more than 5,500 strains) and other Gram-negative rods (more than 2,300 strains).

In This Exercise

Today you will use a multi-test system to run an entire battery of tests designed to identify a member of the *Enterobacteriaceae*.

✓ Materials

Per Class

- ☐ apiweb subscription (https://apiweb.biomerieux.com)

Per Student Group

- ☐ api 20 E identification system for *Enterobacteriaceae* and other Gram-negative rods
- ☐ Sterile 0.85% saline solution
- ☐ Ferric chloride reagent (see Exercise 5-12)
- ☐ Kovac's reagent (see Exercise 5-20)
- ☐ Potassium hydroxide reagent (see Exercise 5-4)
- ☐ Alpha-naphthol reagent (see Exercise 5-4)
- ☐ Sulfanilic acid reagent (see Exercise 5-8)
- ☐ *N,N*-Dimethyl-1-naphthylamine reagent (see Exercise 5-8)
- ☐ Zinc powder (see Exercise 5-8)
- ☐ Oxidase reagent and filter paper (see Exercise 5-7)
- ☐ 3% hydrogen peroxide (see Exercise 5-6)
- ☐ Sterile mineral oil
- ☐ Sterile transfer pipettes
- ☐ Distilled water
- ☐ Streak plates, each containing oxidase-negative, Gram-negative unidentified bacterial colonies
- ☐ api 20 E Results Sheet

Procedure

Lab One

1. Perform a Gram stain on your unknown organism to confirm that it is a Gram-negative rod.
2. Open an incubation box and distribute about 5 mL of distilled water into the honeycombed wells of the tray to create a humid atmosphere (Fig. 5.94).
3. Record the unknown number on the elongated flap of the tray.
4. Remove the strip from its packaging and place it in the tray.
5. Perform the oxidase test on a colony identical to the colony that will be tested. If you need help with this, refer to Exercise 5-7. Record the result on the score sheet as the 21st test.
6. Using a sterile Pasteur pipette, remove a single, well-isolated colony from the plate (Fig. 5.95) and fully emulsify it in a tube of suspension medium or sterile 0.85% saline (Fig. 5.96). Try not to pick up any agar.
7. Using the same pipette, fill both tube and cupule of test CIT, VP, and GEL with bacterial suspension (Fig. 5.97). Fill only the tubes (not the cupules) of all other tests.
8. Overlay the ADH, LDC, ODC, H_2S, and URE microtubes by filling the cupules with sterile mineral oil using a sterile transfer pipette.
9. Close the incubation box and incubate at 35 ± 2°C for 24 hours. (*Note*: If the tests cannot be read at 24 hours, have someone place the incubation box in a refrigerator until the tests can be read.)

5.94 Add Water to Honeycombed Wells ♦ The purpose of the water is to humidify the strip during incubation. Spread the water as uniformly as possible in the honeycomb, but it is not necessary to add identical amounts to each well.

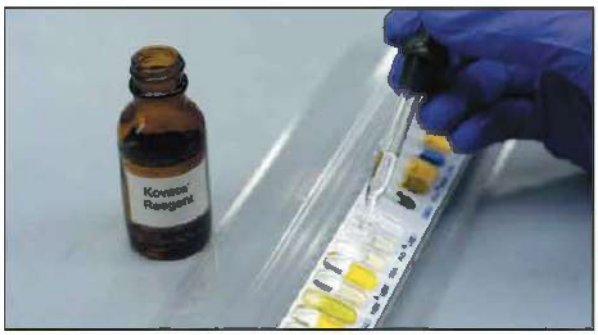
5.95 Pick a Colony ♦ Being careful not to disturb the agar surface, pick up one isolated colony with a sterile transfer pipette.

5.96 Emulsify the Colony in Sterile Saline ◆ Emulsify the colony in 5 mL sterile 0.85% saline. Do this by filling and emptying the pipette several times until uniform turbidity is achieved in the solution.

5 Lab Two

1. Refer to Table 5-36 (Table 2 on the package insert) as you examine the test results on the strip.
2. Record all spontaneous reactions on the Result Sheet. (Spontaneous reactions are reactions completed without the addition of reagents.)
3. **TDA (Tryptophan Deaminase):** Add 1 drop of ferric chloride to the TDA microtube (Fig. 5.98). A dark-brown color indicates a positive reaction to be recorded on the Result Sheet.
4. **VP (Voges–Proskauer):** Add 1 drop of potassium hydroxide reagent and 1 drop of alpha-naphthol reagent to the VP microtube. A pink or red color indicates a positive reaction to be recorded on the Result Sheet. If a slightly pink color appears in 10 to 12 minutes, the reaction should be considered negative.
5. **NIT (Nitrate Reduction) done in GLU microtube:** Add 2 drops of sulfanilic acid reagent and 2 drops of N,N-dimethyl-1-naphthylamine reagent to the GLU microtube. Wait 2 to 3 minutes. A red color indicates a positive reaction ($NO_3 \rightarrow NO_2$). Enter this on the Result Sheet as positive. A yellow color at this point is inconclusive because the nitrate may have been reduced to nitrogen gas ($NO_3 \rightarrow N_2$, sometimes evidenced by gas bubbles). If the substrate in the tube remains yellow after adding reagents, add 2 mg to 3 mg of zinc powder to the tube. A yellow tube after 5 minutes is positive for N_2 and is recorded on the Result Sheet as positive. If the test turns pink-red after the addition of zinc, this is a negative reaction; the nitrates *still present* in the tube have been reduced by the zinc.

5.97 Inoculate the Tubes with the Suspension ◆ Inoculate the tubes by placing the pipette at the side of the tube and gently filling each with the suspension. If necessary to avoid creating bubbles, tilt the strip slightly. Tap gently with your index finger if necessary to remove bubbles.



5.98 Add Reagents after Incubation ◆ Follow the instructions in the text.

6. **IND (Indole Production):** Add 1 drop of Kovac's reagent to the IND microtube. Wait 2 minutes. A red ring indicates a positive reaction to be recorded on the Result Sheet.
7. Add the positive results within each group on the Result Sheet, using the values given, and enter the number in the circle below each group's results (Fig. 5.92).
8. Access the apiweb database: https://apiweb.biomerieux.com. Locate the seven-digit code and identify your organism. Enter the name on the Result Sheet and tape it on the data sheet, page 447. Answer the questions.
9. Properly dispose of the api test strip.

References

api 20 E Identification System for *Enterobacteriaceae* and Other Gram-Negative Rods package insert.

TABLE **5-36** api 20 E Results and Interpretations

Table of Results					
#	Test	Substrate/Activity	Result	Interpretation	Symbol
1	ONPG	O-nitophenyl-β-D-galactopyranoside	Yellow[a]	Organism produces β-galactosidase	+
			Colorless	Organism does not produce β-galactosidase	–
2	ADH	Arginine	Red/orange	Organism produces arginine dehydrolase	+
			Yellow	Organism does not produce arginine dehydrolase	–
3	LDC	Lysine	Red/orange[b]	Organism produces lysine decarboxylase	+
			Yellow	Organism does not produce lysine decarboxylase	–
4	ODC	Ornithine	Red/orange[b]	Organism produces ornithine decarboxylase	+
			Yellow	Organism does not produce ornithine decarboxylase	–
5	CIT	Sodium thiosulfate	Blue-green/blue[c]	Organism utilizes citrate as sole carbon source	+
			Pale green/yellow	Organism does not utilize citrate	–
6	H_2S	Sodium thiosulfate	Black	Organism reduces sulfur	+
			Colorless/grayish	Organism does not reduce sulfur	–
7	URE	Urea	Red/orange[b]	Organism produces urease	+
			Yellow	Organism does not produce urease	–
8	TDA	Tryptophan	Brown-red	Organism produces tryptophan deaminase	+
			Yellow	Organism does not produce tryptophan deaminase	–
9	IND	Tryptophan	Red ring	Organism produces indole	+
			Yellow	Organism does not produce indole	–
10	VP	Creatine	Pink/red[d]	Organism produces acetoin	+
		Sodium pyruvate	Colorless	Organism does not produce acetoin	+
11	GEL	Kohn's charcoal gelatin	Diffusion of black pigment	Organism produces gelatinase	+
			No black pigment diffusion	Organism does not produce gelatinase	–
12	GLU	Glucose[e]	Yellow	Organism ferments glucose	+
			Blue/blue-green	Organism does not ferment glucose	–
13	MAN	Mannitol[e]	Yellow	Organism ferments mannitol	+
			Blue/blue-green	Organism does not ferment mannitol	–
14	INO	Inositol[e]	Yellow	Organism ferments inositol	+
			Blue/blue-green	Organism does not ferment inositol	–
15	SOR	Sorbitol[e]	Yellow	Organism ferments sorbitol	+
			Blue/blue-green	Organism does not ferment sorbitol	–

(Continues)

TABLE 5-36 **api 20 E Results and Interpretations** *(Continued)*

Table of Results					
#	Test	Substrate/Activity	Result	Interpretation	Symbol
16	RHA	Rhamnose[a]	Yellow	Organism ferments rhamnose	+
			Blue/blue-green	Organism does not ferment rhamnose	–
17	SAC	Sucrose (saccharose)[a]	Yellow	Organism ferments sucrose	+
			Blue/blue-green	Organism does not ferment sucrose	–
18	MEL	Melibiose[a]	Yellow	Organism ferments melibiose	+
			Blue/blue-green	Organism does not ferment melibiose	–
19	AMY	Amygdalin[a]	Yellow	Organism ferments amygdalin	+
			Blue/blue-green	Organism does not ferment amygdalin	–
20	ARA	Arabinose[a]	Yellow	Organism ferments arabinose	+
			Blue/blue-green	Organism does not ferment arabinose	–
21	OX	Separate test done on paper test strip	Violet	Organism produces cytochrome-oxidase	+
			Colorless	Organism does not produce cytochrome-oxidase	–
22	GLU	Potassium nitrate	Red after addition of reagents	Organism reduces nitrate to nitrite	+
			Yellow after addition of reagents	Organism does not reduce nitrate to nitrite	–
			Yellow after addition of zinc	Organism reduces nitrate to N_2 gas	+
			Orange-red after addition of zinc	Organism does not reduce nitrate	–
23	MOB	Motility medium or wet mount slide	Motility	Organism is motile	+
			Nonmotility	Organism is not motile	–
24	McC	MacConkey medium	Growth	Organism is probably *Enterobacteriaceae*	+
			No growth	Organism is not *Enterobacteriaceae*	–
25	OF	Glucose	Yellow under mineral oil	Organism ferments glucose	+
			Green under mineral oil	Organism does not ferment glucose (not *Enterobacteriaceae*)	–
			Yellow without mineral oil	Organism either ferments glucose or utilizes it oxidatively	+
			Green without mineral oil	Organism does not utilize glucose (not *Enterobacteriaceae*)	–

[a] Very pale yellow is also positive
[b] Orange after 36 hours is negative
[c] Reading made in the capsule
[d] Slightly pink color after 5 minutes is negative
[e] Fermentation begins in the lower portion of the tube; oxidation begins in the capsule

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-29

api 20 E Identification System for *Enterobacteriaceae* and Other Gram-negative Rods

OBSERVATIONS AND INTERPRETATIONS

1 Tape your api 20 E Result Sheet here.

QUESTIONS

1 *Why is it important to perform the reagent tests last?*

2 *In clinical applications of this test system, reagents are added only if the glucose (oxidation/fermentation) test result is yellow or at least three other tests are positive. If these conditions are not met, a MacConkey agar plate is streaked and additional tests are performed confirming glucose metabolism, nitrate reduction, and motility. Why do you think this is so? Be specific.*

3 *Suppose, after 24 hours incubation, you notice no growth in the tubes containing mineral oil. Assuming that it is behaving properly under these conditions, what do you know about the organism and what predictions can you safely make about its performance in the decarboxylase tests, fermentation tests, and nitrate reduction test? Is it a member of* Enterobacteriaceae*?*

EnteroPluri–*Test* (Enterotube® II)

EXERCISE 5-30

Preface

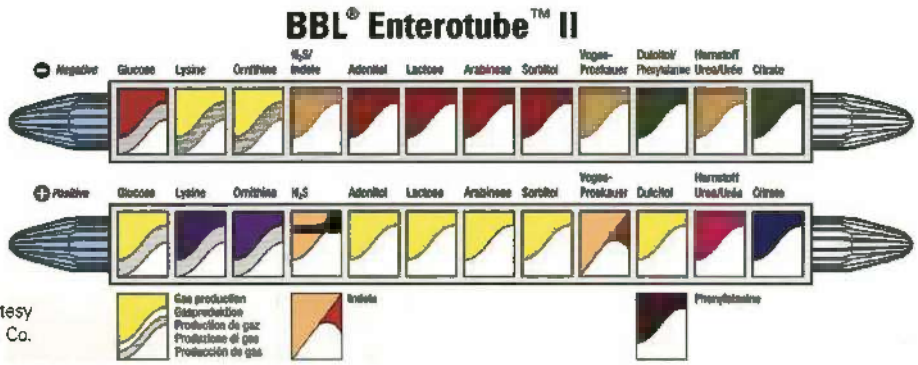
For many years, the Enterotube® II was a popular multi-test system designed to identify enteric bacteria and other select oxidase-negative, Gram-negative bacteria. While there may still be unused Enterotubes® in college labs around the country, as far as we can determine it is no longer available for purchase. However, a virtually identical system called EnteroPluri–*Test* is manufactured by the Italian company Liofilchem® and sold by various vendors in the United States[1]. This lab exercise is still written for the Enterotube® II, but it will also work for the EnteroPluri–*Test* system. For clarity during this transitional time, we refer only to Enterotube® II.

[1] A quick web search located Hardy Diagnostics and Carolina Biological Supply Company as sources of the EnteroPluri–*Test* and EnteroPluri Code Book.

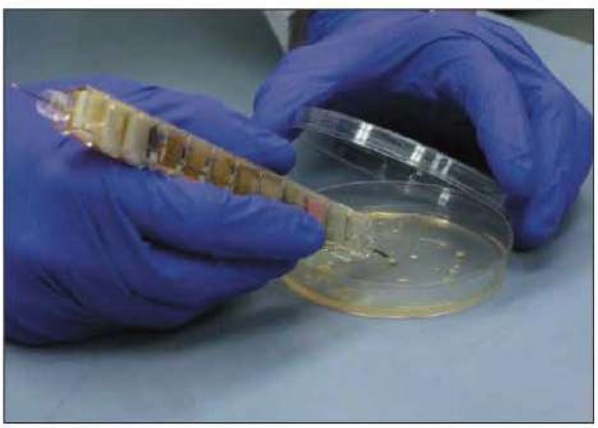
Theory

The Enterotube® II is a multiple test system designed to identify enteric bacteria based on: glucose, adonitol, lactose, arabinose, sorbitol, and dulcitol fermentation, lysine and ornithine decarboxylation, sulfur reduction, indole production, acetoin production from glucose fermentation, phenylalanine deamination, urea hydrolysis, and citrate utilization.

The Enterotube® II, as diagramed in Figure 5.99, is a tube containing 12 individual chambers. Inside the tube, running lengthwise through its center, is a removable wire. After the end-caps are removed (aseptically), one end of the wire is touched to an isolated colony on a streak plate and drawn back through the tube to inoculate the media in each chamber (Figs. 5.100 through 5.104).



5.99 Enterotube® II Test Result Diagram ◆ Illustration courtesy Becton Dickinson and Co.



5.100 Pick a Colony ◆ Using the sterile tip of the wire, aseptically remove growth from a colony on the agar surface. Do not dig into the agar. The inoculum should be large enough to be visible. Remember, there must be enough to inoculate all 12 compartments.

5.101 Pull Wire Through ◆ After removing the caps and placing them on the disinfectant-soaked towel, loosen the wire by turning it slightly. While continuing to rotate it withdraw the wire until its tip is inside the last compartment (glucose).

5.102 Reinsert Wire ♦ Slide the wire back into the Enterotube® II until you can see the tip inside the citrate compartment. The notch in the wire should be lined up with the end of the tube nearest the glucose compartment.

5.103 Break Wire ♦ Bend the wire until it breaks off at the notch.

5

5.104 Puncture Air Inlets ♦ Using the broken wire, puncture the plastic covering the eight air inlets on the back side of the tube.

5.105 Add Reagents after Incubation ♦ Use a needle and syringe to add the reagents through the plastic film on the flat side of the tube. Do this with the tube in a test tube rack and the glucose end pointing down. The indole test should be done only after other tests have been read. Add the Voges-Proskauer reagents only if directed to do so by the CCIS.

After 18 to 24 hours' incubation, the results are interpreted, the indole test is performed (Fig. 5.105), and the tube is scored on an Enterotube® II Results Sheet (Fig. 5.106). As shown in the figure, the combination of positives entered on the score sheet results in a five-digit numeric code. This code is used for identification in the Enterotube® II Computer Coding and Identification System (CCIS) (Fig. 5.107).

The CCIS is a master list of all enterics and their assigned numeric codes. In most cases, the five-digit number applies to a single organism, but when two or more species share the same code, a confirmatory VP test is performed to further differentiate the organisms.

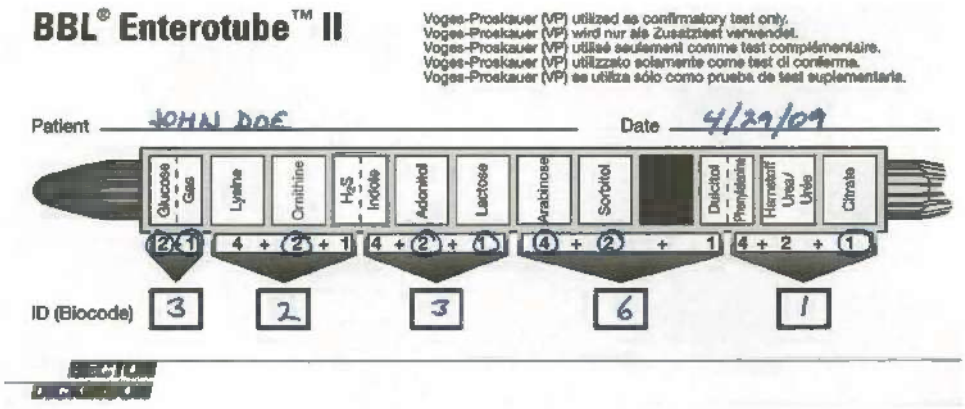
An Enterotube® II before and after inoculation is shown in Figure 5.108.

Application

The Enterotube® II is a multiple test system used for rapid identification of bacteria from the family *Enterobacteriaceae*.

In This Exercise

You will use a multi-test system to run an entire battery of tests designed to identify a member of the *Enterobacteriaceae*.



5.106 BBL™ Enterotube® II Result Sheet ◆ This is the result sheet for the tube in Figure 5.108B. The ID value obtained was 32361, which identifies the organism as *Enterobacter aerogenes*.

5.107 Sample of the Enterotube® II Computer Coding and Identification System ◆ The five-digit numbers on the Enterotube® II Results Pad are matched against the database in the CCIS (or EnteroPluri–*Test* Codebook) to get identification.

ID VALUE	ORGANISM IDENTIFICATION	ATYPICAL TESTS	CONFIRMATORY TESTS				
			MAL	TRE			
20406	PROTEUS VULGARIS	H2S-	+	V(30%)			
	MORGANELLA MORGANII	ORN-	-	-			
	PROVIDENCIA STUARTII	PAD-	-	+			
20407	PROVIDENCIA STUARTII	NONE					
			SHA				
20410	*SHIGELLA SEROGROUPS A, B OR C	DUL+	+				
			SHA	ADA			
20420	*SHIGELLA SEROGROUPS A, B OR C	SOR+	+	-			
	ESCHERICHIA COLI (AD)	ARA-	-	+			
20425	PROVIDENCIA STUARTII	SOR+					
20427	PROVIDENCIA STUARTII	SOR+					
20430	ESCHERICHIA COLI (AD)	ARA-					
			SHA	ADA	MOT	VP	YEL
20440	*SHIGELLA SEROGROUPS A, B OR C	NONE	+	-	-	-	-
	ESCHERICHIA COLI (AD)	SOR-	-	+	-	-	-
	ENTEROBACTER AGGLOMERANS	IND+	-	-	+	V(70%)	+(75%)
20441	ENTEROBACTER AGGLOMERANS	IND+					

5.108 Enterotube® II Test Results ◆ (A) Shown is an uninoculated tube. (B) This tube was inoculated with *Enterobacter aerogenes* and incubated for 24 hours. This *E. aerogenes* strain shows atypical negative results for lysine decarboxylase; after an additional 24 hours incubation the lysine medium turned purple (+).

✓ Materials

Per Class (based on system used)

- ☐ Enterotube® II Computer Coding and Identification System (CCIS) identification booklet from Becton Dickinson Microbiology Systems
- ☐ EnteroPluri–*Test* Code Book

Per Student Group

- ☐ Becton Dickinson Microbiology Systems' Enterotube® II Identification System for *Enterobacteriaceae*
- ☐ EnteroPluri–*Test* System
- ☐ Kovac's reagent
- ☐ VP reagents (KOH and α-naphthol)
- ☐ Needles and syringes or disposable pipettes for addition of reagents
- ☐ Streak plate containing colonies of an unknown enteric (appropriate to facilities and course level)

5

■ Reagent Recipes

Indole Test

Kovac's Reagent

• Amyl alcohol	75.0 mL
• Hydrochloric acid, concentrated	25.0 mL
• *p*-dimethylaminobenzaldehyde	5.0 g

VP Test

KOH Reagent

• Potassium hydroxide	20.0 g
• Distilled water to bring volume to	100.0 mL

α-Naphthol Reagent

• α-naphthol	5.0 g
• Absolute ethanol to bring volume to	100.0 mL

Procedure

Lab One

1. Perform a Gram stain on your isolate to verify that it is a Gram-negative rod.
2. Place a paper towel on the tabletop and soak it with disinfectant.
3. Remove the blue cap and then the white cap from the Enterotube® II, being careful not to contaminate the sterile wire tip. Place the caps open end down on the paper towel.
4. Aseptically remove a large amount of growth from one of the plated colonies (Fig. 5.100). (This presumes your streak plate has only one organism on it. If not, you will need to inoculate with the same colony you Gram stained.) Try not to remove any of the agar with it.
5. Grasp the looped end of the wire and, while turning, gently pull it back through all of the Enterotube® II compartments (Fig. 5.101). You do not have to completely remove the wire, but be careful to pull it back far enough to inoculate the last compartment.
6. Using the same turning motion as described above, slide the wire back into the tube until the notch in the wire lines up with the end of the tube (Fig. 5.102).
7. Bend the wire at the notch until it breaks off (Fig. 5.103).
8. Locate the air inlets on the side of the tube opposite the Enterotube® II label. Using the removed piece of inoculating wire, puncture the plastic membrane in the adonitol, lactose, arabinose, sorbitol, Voges-Proskauer, dulcitol/PA, urea, and citrate compartments (Fig. 5.104). These openings will create the necessary aerobic conditions for growth. Be careful not to perforate the plastic film covering the flat side of the tube.
9. Discard the wire in disinfectant or a sharps container and replace the Enterotube® II caps.
10. Incubate the tube lying on its flat surface (or vertically with glucose on top, if using EnteroPluri–*Test*) at 35 ± 2°C for 18 to 24 hours. (*Note*: If the tube cannot be read at 24 hours, have someone place it in a refrigerator until it can be read.)

Lab Two

1. Examine the tube and circle the appropriate positive results on the Enterotube® II Results Sheet. ***Note***: while the tests are listed in the same order, the EnteroPluri–*Test* scoring sheet differs slightly from the Enterotube® II's in their grouping, as shown below.

   Group 1: Glucose, Gas, and Lysine
   Group 2: Ornithine, H_2S, and Indole
   Group 3: Adonitol, Lactose, and Arabinose
   Group 4: Sorbitol, VP, and Dulcitol
   Group 5: Phenylalanine, Urea, and Citrate

2. *After* circling all positive test results on the Enterotube® II Results Sheet, place the tube in a rack with the glucose compartment on the bottom. Puncture the plastic membrane of the H_2S/Indole compartment and (with a needle and syringe or disposable pipette) add one or two drops Kovac's reagent to the compartment (Fig. 5.105).

3. Observe the compartment for the formation of a red color (+) within 10 seconds. Record the result on the Enterotube® II Results Sheet.

4. Add the numbers in each bracketed section on the Enterotube® II Results Sheet to obtain a five-digit number. Find the number in the CCIS. If not directed to perform a VP test, record the name of your organism on the score sheet and skip to step 6 below. If directed to run a confirmatory Voges-Proskauer test, proceed to step 5. (EnteroPluri–*Test* has you run the VP at this point.)

5. Puncture the plastic membrane of the VP compartment and (with a needle and syringe or disposable pipette) add two drops of the KOH reagent and three drops of the α-Naphthol reagent. Observe for the formation of red color within 20 minutes.

6. Interpret the result using the CCIS or EnteroPluri–*Test* Codebook. The codes obtained are not interchangeable between the two systems.

7. Discard the Enterotube II® in an appropriate autoclave container.

8. Enter all results on the data sheet, page 455 and answer the questions.

References

Enterotube® II Identification System for *Enterobacteriaceae* package insert.

Enterotube® II Computer Coding and Identification System.

EnteroPluri–*Test* package insert.

EnteroPluri–*Test* Codebook.

TABLE 5-37 Enterotube® II Results and Interpretations

Table of Results				
Compartment	**Test**	**Result**	**Interpretation**	**Symbol**
1	Glucose	Red	Organism does not ferment glucose	–
		Yellow, wax not lifted	Organism ferments glucose to acid	+
		Yellow, wax lifted	Organism ferments glucose to acid and gas	+
2	Lysine	Purple	Organism decarboxylates lysine	+
		Yellow	Organism does not decarboxylate lysine	–
3	Ornithine	Purple	Organism decarboxylates ornithine	+
		Yellow	Organism does not decarboxylate ornithine	–
4	H_2S	Beige	Organism does not reduce sulfur	–
		Black	Organism reduces sulfur	+
	Indole	Red after Kovac's reagent	Organism produces indole	+
		No red after Kovac's reagent	Organism does not produce indole	–
5	Adonitol	Red	Organism does not ferment adonitol	–
		Yellow	Organism ferments adonitol	+
6	Lactose	Red	Organism does not ferment lactose	–
		Yellow	Organism ferments lactose	+
7	Arabinose	Red	Organism does not ferment arabinose	–
		Yellow	Organism ferments arabinose	+
8	Sorbitol	Red	Organism does not ferment sorbitol	–
		Yellow	Organism ferments sorbitol	+
9	VP	Colorless	Organism does not produce acetoin	–
		Red	Organism produces acetoin	+
10	Dulcitol	Green	Organism does not ferment dulcitol	–
		Yellow	Organism ferments dulcitol	+
	PA	Green	Organism does not deaminate phenylalanine	–
		Black-smoky-gray	Organism deaminates phenylalanine	+
11	Urea	Beige	Organism does not hydrolyze urea	–
		Red-purple	Organism hydrolyzes urea	+
12	Citrate	Green	Organism does not utilize citrate	–
		Blue	Organism utilizes citrate	+

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-30

EnteroPluri–*Test* (Enterotube® II)

OBSERVATIONS AND INTERPRETATIONS

1 Tape your BBL® Enterotube® II Result Sheet here.

2 Enter the results from the CCIS booklet below.

CCIS Five-Digit Code	Possible Organisms	VP Result + / –	Identified Organism

QUESTIONS

1 *Fecal coliforms such as* Escherichia coli, Enterobacter aerogenes, *and* Klebsiella pneumoniae *are enterics that ferment lactose to acid and gas at 35°C within 48 hours. For most strains of these organisms, the table below summarizes reactions in the Enterotube® II. Fill in the missing information and, using colored pencils, fill in the appropriate colors for each positive test.*

	Glu	Gas	Lys	Orn	H_2S	Ind	Adon	Lact	Arab	Sorb	VP	Dulc	PA	Urea	Citrate
E. coli			+	−	−	+	+		+	+	−	−	−	−	−
E. aerogenes			+	+	−	−	−		+	+	+	−	−	−	+
K. pneumoniae			+	−	−	−	−		+	+	+	−	−	+	+

2 *Based on the information above, enter the five-digit codes for the three organisms.*

E. coli _____ *E. aerogenes* _____ *K. pneumoniae* _____

5

3 *Examine the following table. Fill in the color reactions for each test based on the ID value given.*

ID Value	Glucose/ Gas	Lysine	Ornithine	H_2S/ Indole	Adonitol	Lactose	Arabinose	Sorbitol	VP	Dulcitol/ PA	Urea	Citrate
31122												
27345												
04004												
05511												

4 *Which of the four organisms are not members of* Enterobacteriaceae? *Why?*

_____

_____

5 *Which of the four ID values is questionable? Why?*

_____

_____

_____

Bacterial Unknowns Project

The following exercise is actually a term project to be done when you have completed Sections One through Five. These sections prepare you with the skills needed to successfully do what professional laboratory microbiologists do daily—that is, *isolate* from mixed culture, *grow* in pure culture, and *identify* unknown species of bacteria. Scared? Don't worry. You will not be asked to perform any task not introduced in previous sections. If you have any uncertainty regarding performance of these activities, refer to the appropriate exercises.

Although this project may seem intimidating, it is manageable if you don't try to do too much too fast. Take your time and think about what you want to do next, based on earlier results. Use the information from the lessons learned in Exercise 3-13, and closely follow the procedures listed.

This exercise gives you a unique opportunity (and responsibility) to set your own course in a learning project. Take advantage of it, because there is great fun and satisfaction to be had. Our experience tells us that students traditionally do one of three things. They

1. treat the project as a fun puzzle to solve,
2. become completely stressed out and hate every minute of it,
3. get lost early on and, for whatever reason, don't ask for help until it is too late to receive meaningful assistance.

Many of you will be nervous, perhaps even confused at first. Your instructor understands this. It is normal and actually can help you to focus if you use it in a positive way. For your success and overall satisfaction with this project, *ask you instructor for help if you need it.* Happy hunting! ◆

EXERCISE 5-31

Bacterial Unknowns Project

In This Exercise

You will be given a mixture of two bacteria in broth culture. Your first job is to get the bacteria isolated and growing in pure culture, using the techniques of Sections Two and Four. Next, you will employ Section Three techniques as you perform Gram stains on each to determine Gram reaction, as well as cell morphology and size. Then, results from the differential biochemical tests of Section Five will lead you to identification of your unknowns. And, of course, all work must be done safely and aseptically using the methods covered in Lab Safety (Introduction) and Section One.

As in Exercise 3-13 (Morphological Unknown), you will be expected to keep accurate records of all activities, including mistakes and unexpected or equivocal results. You also will be expected to construct a flowchart for each to show the tests you ran and the organisms eliminated by each until you have eliminated all but your unknown. The flowchart is a visual presentation of your thought processes in solving this problem.

You will be advised by your instructor of the possibility for your two unknowns to include any BSL-2 organisms. If there is the possibility, and because one or both of your unknowns in the mixture may be BSL-2, you must follow BSL-2 precautions throughout this project. Review these in the Introduction, pages 5 and 6. If you are unsure what to do in a particular instance after reviewing the precautions, ask your instructor for help.

✓ Materials

Recommended organisms (to be mixed in pairs in a broth by the lab technician immediately prior to use—generally, a few drops of the Gram negative added to an overnight culture of the Gram positive provides an appropriate ratio for isolation and growth).

Gram positives

- ☐ *Bacillus cereus*
- ☐ *Bacillus coagulans*

- ☐ *Corynebacterium xerosis*
- ☐ *Enterococcus faecalis* (BSL-2)
- ☐ *Kocuria rosea*
- ☐ *Lactobacillus plantarum*
- ☐ *Lactococcus lactis*
- ☐ *Micrococcus luteus*
- ☐ *Mycobacterium smegmatis* (BSL-2)
- ☐ *Staphylococcus aureus* (BSL-2)
- ☐ *Staphylococcus epidermidis*

Gram negatives

- ☐ *Aeromonas hydrophila* (BSL-2)
- ☐ *Alcaligenes faecalis* (BSL-2)
- ☐ *Chromobacterium violaceum* (BSL-2)
- ☐ *Citrobacter amalonaticus*
- ☐ *Enterobacter aerogenes*
- ☐ *Erwinia amylovora*
- ☐ *Escherichia coli*
- ☐ *Hafnia alvei*
- ☐ *Moraxella catarrhalis* (BSL-2)
- ☐ *Morganella morganii* (BSL-2)
- ☐ *Neisseria sicca*
- ☐ *Proteus mirabilis* (BSL-2)
- ☐ *Pseudomonas aeruginosa* (BSL-2)
- ☐ Appropriate stains and biochemical media

Procedure

1 Preliminary duties

a Your instructor will tell you which organisms will actually be used based on your lab's inventory. You also will be advised as to the optimum temperature for the specific strain your lab has of each organism.

b Your instructor will tell you which media and stains will be available for testing.

c Working as a class, you will determine the results of each organism for each test available. This information will provide a database of results that you can use to compare against your unknown's results. We recommend running and using these "class controls" in lieu of referring to results in *Bergey's Manual of Systematic Bacteriology* or some other standard reference, for the following reasons:

- As many as 10% of the strains of a species listed as positive or negative on a test give the opposite result.
- Many species have even higher strain variability.
- Not all test results are listed for all organisms.

d Class control tests should be run for the standard times, unless the timing of class sessions makes this impractical. It is imperative that incubation times for tests on unknowns be exactly the same as was used for the controls.

e You are to incubate your class controls at the optimum temperature for each species as given to you by your instructor.

f Class control results will be collected, tabulated, and distributed to each student. Your instructor will provide details on this process, but one option is entering results into an Excel spreadsheet that can be distributed when completed.

2 Isolation of the Unknown

a You will be given a numbered broth containing a *fresh* mixture of two unknown bacteria selected from the list of possible organisms (as modified by your instructor). Enter the number of your unknown on your data sheets, pages 463–466.

b Mix the broth, then streak for isolation on two agar plates. Your instructor will tell you what media are available for use. You may be supplied with an undefined medium, such as tryptic soy agar, or a selective medium, such as phenylethyl alcohol agar or desoxycholate agar. Enter all relevant information concerning your isolation procedures on the data sheets, including date, medium, source of bacteria, type of inoculation, and incubation temperature.

c Incubate one agar plate at 25°C and the other at 35 ± 2°C for at least 24 hours.

d After incubation, check for isolated colonies that have different morphologies. If you have isolation of both, go on to step 3a. If you do *not* have isolation of *either*, continue with step 2e. If you have isolation of only one, go to step 3a for the isolated species and step 2e for the one not isolated. Be sure to enter relevant information on the data sheets.

e If you do not have isolation, follow the advice that best matches your situation. (Be sure to record everything you do in the isolation. Include the date, source of inoculum, medium to which it is being transferred, type of inoculation, and incubation temperature.)

- Look for growth with different colony morphologies, even if they are not separate. If you see different growth, use a portion of each and streak more plates. Then incubate them for

either a shorter time or at a suboptimal temperature, because they grew *too* well the first time.

- If you see only one type of growth, ask for a selective medium that favors growth of the one you are missing. (This will require a Gram stain of the one you do have.) Streak the mixture and incubate again. Also, reincubate your original plate. Some species are slow growers, so their absence may be a result of a slow growth rate.
- After incubation, observe the plate(s) and repeat or go to step 3a, whichever is appropriate. You also may consult with your instructor for guidance in particularly difficult situations.

3 Growing the Unknown in Pure Culture

a Once you have isolation of an unknown, transfer a portion of its colony to an agar slant (e.g., NA, TSA, BHIA) to produce a pure culture. Use the rest of the colony for a Gram stain. (If you do not have enough of the colony left for a Gram stain, do the Gram stain on your pure culture after incubation.)

b After Gram staining, label your pure culture accordingly.

c Enter the following information on your data sheet: optimum growth temperature, colony morphology, cell morphology and arrangement, and cell dimensions. Also, complete the description of your isolation procedure by noting the source of the isolate, the medium to which you are transferring it, and the incubation temperature.

4 Identification of the Unknown

a Follow this procedure for each unknown. Be sure to enter on the data sheet the inoculation and reading dates for each test. Also include the test result and any comments about the test that explain any deviation from standard procedure (e.g., reading tests before or after the accepted incubation time, running a test and not using it in the flowchart, etc.).

b Construct a flowchart from the class control data that divides all the organisms first by Gram reaction, then by cellular morphology. Use the flowchart in Figure 5.109 as a style guide even though the actual organisms you use will be different.

c You will have two options for proceeding at this point. Your instructor will let you know which to use.

(1) Find the group of organisms on the flowchart that matches the results of your unknown. Then, look at your class controls database for *just those organisms*, and choose a test that will divide these organisms into at least two groups. (Also consider your ability to return and read the test after the appropriate incubation time. That is, do not inoculate a 48-hour test on Thursday if you can't get into the lab on Saturday!) Continue the flowchart from the branch with the remaining candidates for your unknown.

(2) Rather than allowing you to choose just any test that works, you may be required to follow some form of standard approach to identification. Your instructor will provide you with relevant information based on the organism inventory you are working with.

d Inoculate the test medium you have chosen, and incubate it for the appropriate time. Use the optimum temperature for growth as given by your instructor. *It is important to run your tests at that optimum temperature because this is the temperature at which the class controls were run.*

e While the test is incubating, you should begin planning what your next test will be. A final decision about the next test cannot be made until you have results from the first, but you can decide which test to run if the result is positive, and which one to run if it is negative. This applies to all stages of the flowchart: Because you will not know if a subsequent test is relevant or not until you have results from the current one, *you should not inoculate a medium until you have those results.* It is really easy: Run one test at a time for each unknown until you have a result for that test. (*Note:* In a clinical situation, where rapid identification of a pathogen may save a patient's life, tests routinely are run concurrently. But remember that correct identification is only one objective of this project. More important is for you to demonstrate an understanding of the logic behind the process and execute it in the most efficient manner.)

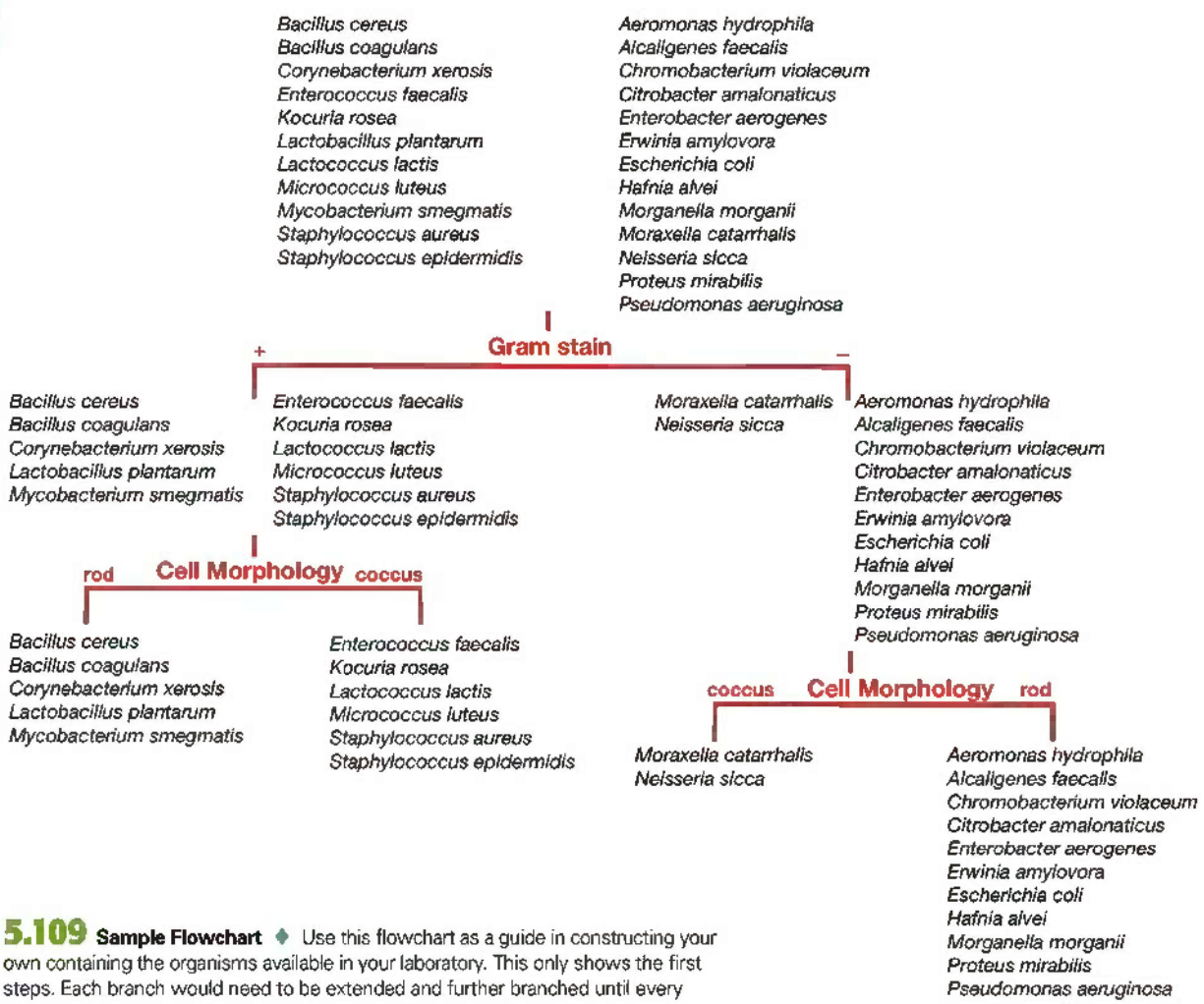
f Repeat the process of inoculating a medium, getting the results, and then choosing a subsequent test until you eliminate all but one organism. This *should* be your unknown. Then continue with step 4g.

g When you have eliminated all but one organism, you will run one more test—the **confirmatory test.** This must be a test that has not been run previously while identifying that organism, but it does not have to differentiate between your provisionally identified organism and the other remaining candidates. It is also nice (but not necessary) if the test you choose gives a positive result. (In general, we have more confidence in positive results than in negative results, because false

positives are usually harder to get than false negatives.) The confirmatory test provides you with the unique opportunity to predict the result before you run the test. If it matches, you are more certain that you have correctly identified your unknown. Continue with step 4j. If it does not match, continue with step 4h.

h If your confirmatory test does not match the result you expected for your unknown, check with your instructor to see where your organism was eliminated incorrectly. In most cases, it will be difficult at this point to determine what was responsible—you, the class controls, or the organism itself. Misidentification may be a result of one or any combination of factors:

- The test procedure could have been done incorrectly by you or the person responsible for running class controls on your organism.
- The test may have been interpreted incorrectly by you or the person responsible for running class controls on your organism.
- The inoculum in your test or the class controls might have been too small to give a positive result in the limited incubation time.
- The wrong organism could have been inoculated at the time of the test or the class controls (many look alike in a tube, and once the label goes on, as far as the microbiologist is concerned, that culture becomes the labeled organism regardless of what is really in there!)



5.109 Sample Flowchart ♦ Use this flowchart as a guide in constructing your own containing the organisms available in your laboratory. This only shows the first steps. Each branch would need to be extended and further branched until every organism in the starting list is alone at the end of a branch.

- For whatever reason, the organism did not react "correctly" during your test or during the class controls.

i Based on your instructor's advice, you will do one of the following:

- Rerun the test where you incorrectly eliminated your unknown (and perhaps rerun the test on the remaining organisms to check the class control results), or
- Rerun the test where you incorrectly eliminated your unknown without running the controls again, or
- Eliminate the problematic test from your flowchart, but use the other tests you have already done. If these don't allow you to identify your unknown, more tests will need to be run. Continue with step 4f.

(*Note*: There will undoubtedly be some "mistakes" uncovered in the class controls as they get repeated. These have to be reported to the class so classmates can incorporate the correct information into their flowcharts.)

j When you have correctly identified your unknown, complete the data sheet, and turn it in. Your instructor will advise you as to the point value of each section, the grading scale, and any other items that are required.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-31

Bacterial Unknowns Project: Gram-Positive Unknown

OBSERVATIONS AND INTERPRETATIONS

Unknown Number _____

Isolation Procedure Record all activities associated with isolation of your Gram-positive organism—from mixed culture to pure culture. Always include the date, source of inoculum, destination, incubation temperature, and any other relevant information. Also, make note of transfers made to keep your pure culture fresh. This log must be kept current.

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Preliminary Observations

Colony Morphology (include medium and incubation temperature) _____

_____

_____

Gram Stain _____ Cell Dimensions _____ Optimum Temperature _____

Cellular Morphology and Arrangement _____

Differential Tests

Include all information through the confirmatory test. This log must be kept current.

Test #1: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #2: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #3: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #4: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

5 **Test #5:** _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #6: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #7: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #8: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #9: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #10: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #11: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #12: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

My Unknown is: _____ Rerun[1]: _____

Rerun: _____

Rerun: _____

[1] Your instructor will write what tests to rerun in this space if you misidentify your unknown.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 5-31

(continued)

Bacterial Unknowns Project: Gram-Negative Unknown

OBSERVATIONS AND INTERPRETATIONS

Unknown Number _____

Isolation Procedure Record all activities associated with isolation of your Gram-negative organism—from mixed culture to pure culture. Always include the date, source of inoculum, destination, incubation temperature, and any other relevant information. Also, make note of transfers made to keep your pure culture fresh. This log must be kept current.

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Preliminary Observations

Colony Morphology (include medium and incubation temperature) _____

_____

_____

Gram Stain _____ Cell Dimensions _____ Optimum Temperature _____

Cellular Morphology and Arrangement _____

Differential Tests

Include all information through the confirmatory test. This log must be kept current.

Test #1: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #2: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #3: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #4: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

5 **Test #5:** _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #6: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #7: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #8: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #9: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #10: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #11: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #12: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

My Unknown Is: _____ Rerun[2]: _____

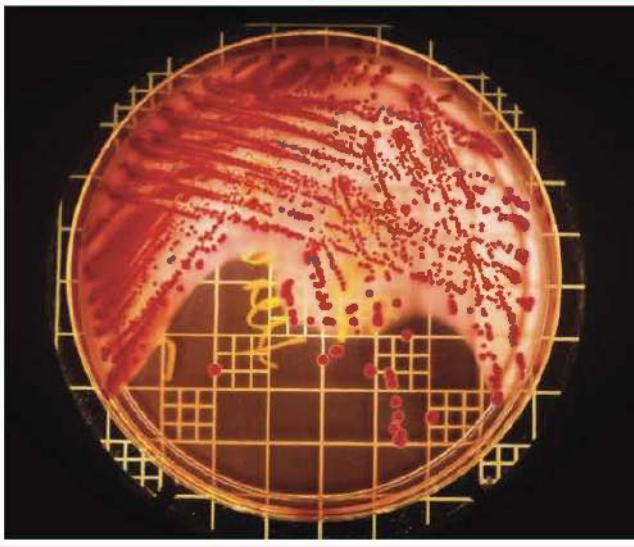
Rerun: _____

Rerun: _____

[2] Your instructor will write what tests to rerun in this space if you misidentify your unknown.

SECTION 6

Quantitative Techniques



Quantitative techniques in microbiology usually consist of counting cells or counting colonies formed by cells growing on agar plates. A typical result is a close estimate of the number of cells (or viruses) in one milliliter of sample—the population density, or **cell density**. Knowledge of microbial **population density** is an important part of many areas of microbiology. Food and environmental microbiologists use population density for food production as well as the detection of food or water contamination. Medical microbiologists manipulate population density for use in standardized tests. Many researchers use population density to measure the effect of varying nutritional or environmental conditions. Industrial microbiologists maintain microbial populations at optimum levels in large-scale fermenters for the manufacture of many products, including enzymes, antibiotics, beer, and wine.

In this section we include five bacterial counting techniques and one exercise to measure a viral population. One technique involves directly counting cells, and the other four involve plate counts. As you will see, all techniques involve dilutions and all require calculations to determine the *original* population density.

The standard dilution procedure used in microbial counts is the **serial dilution**. A serial dilution is a series of individual measured transfers, starting with an undiluted sample, one tube to the next, each reducing the cell density by a known amount, until it becomes dilute enough to be used in an appropriate counting procedure (Fig. 6.1). The advantage of a serial dilution is that it not only reduces the cell density to a manageable size, but does so systematically so that each dilution tube contains a *known* volume of the undiluted original.

An important term you will see in the following exercises is colony forming units, or CFU. As you recall, it takes a minimum of one cell to grow into a colony, but many cells grow in pairs, chains, or clusters, so we cannot equate colonies on the plate with cells deposited there in the sample. Therefore, the count is usually recorded in CFU, because we do not know with certainty how many cells actually produced each colony. (***Note***: Viral samples are recorded in PFU [plaque forming units], which will be explained fully in Exercise 6-5.)

As you proceed through the exercises in this section, you will gain understanding and proficiency with serial dilutions. Because these exercises involve math, some of you will be a little (or a lot) intimidated. Take heart; these calculations are much simpler than they appear! If you are nervous about the math, we encourage you to see your instructor and to work as many practice problems as you can get. Before you know it, you will see these equations as a welcome solution to the very real problem of how to manage otherwise unmanageable numbers!

Most of the quantitative techniques in this section were originally designed for measurements and calculations in milliliters. Many school laboratories now are equipped with digital micropipettes that have the ability to deliver volumes as small as 1.0 microliter (1.0 µl = 0.001 mL; or 1.0 mL = 1,000 µL). To accommodate schools with modest budgets and for ease of instruction, we have written the following exercises for measurements and calculations in milliliters. Alternative procedures, adapted for digital micropipettors, are included in Appendix F.

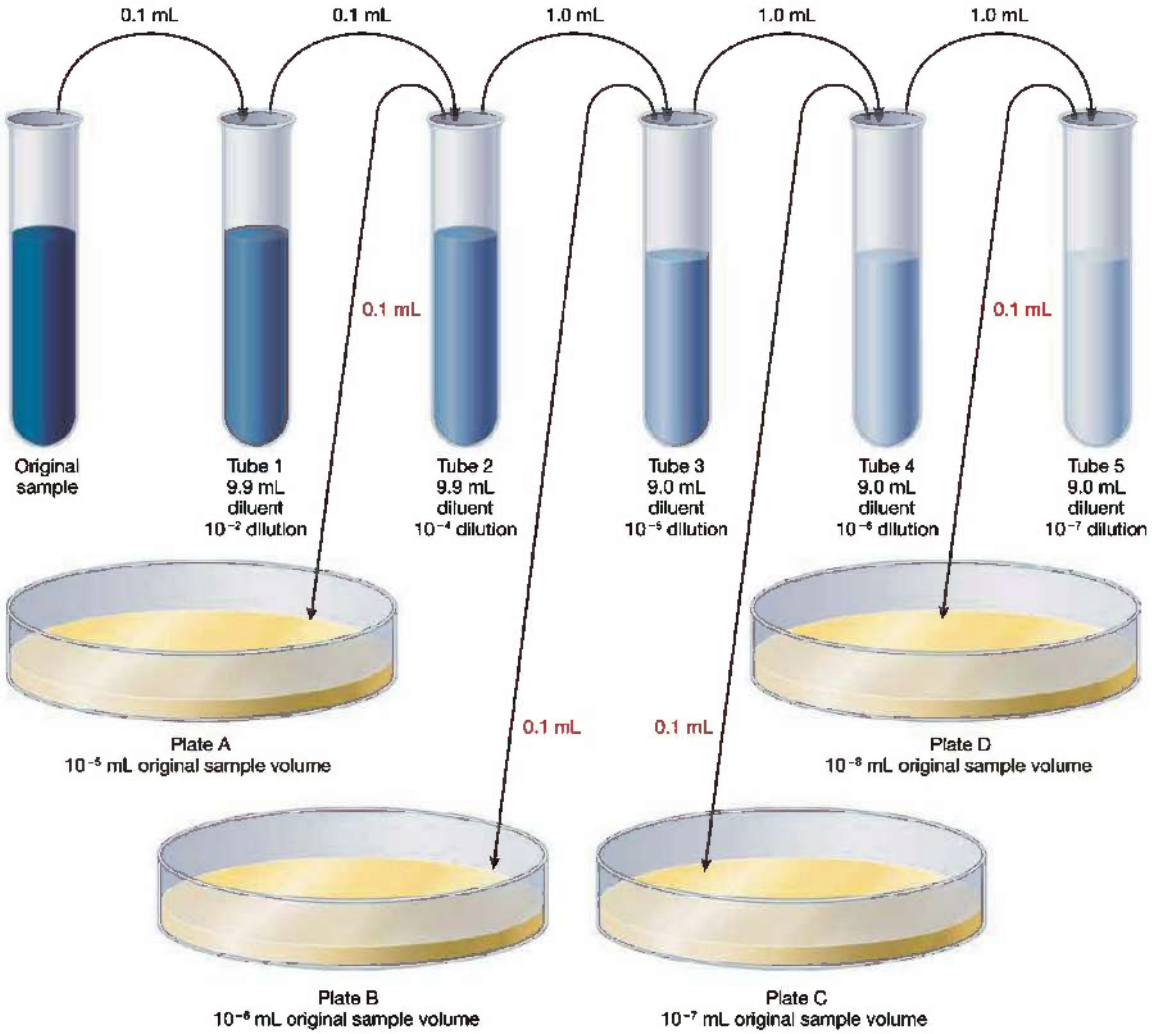
Standard Plate Count (Viable Count)

EXERCISE 6-1

Theory

The standard plate count is a procedure that allows microbiologists to estimate the population density in a liquid sample by plating a very dilute portion of that sample and counting the number of colonies it produces. The inoculum that is transferred to the plate contains a *known* proportion of the original sample because it is the product of a **serial dilution.**

As shown in Figure 6.1, a serial dilution is simply a series of controlled transfers down a line of **dilution blanks** (tubes containing a known volume of sterile diluent—water, saline, or buffer). The series begins with a sample containing an unknown concentration (density) of cells and ends with a very dilute mixture containing only a few—or no—cells. Each dilution blank in the series


0.1 mL
0.1 mL
1.0 mL
1.0 mL
1.0 mL
0.1 mL
0.1 mL
Original sample
Tube 1 9.9 mL diluent 10^{-2} dilution
Tube 2 9.9 mL diluent 10^{-4} dilution
Tube 3 9.0 mL diluent 10^{-5} dilution
Tube 4 9.0 mL diluent 10^{-6} dilution
Tube 5 9.0 mL diluent 10^{-7} dilution
0.1 mL
0.1 mL
Plate A 10^{-5} mL original sample volume
Plate D 10^{-8} mL original sample volume
Plate B 10^{-6} mL original sample volume
Plate C 10^{-7} mL original sample volume

6.1 Serial Dilution Procedural Diagram ◆ This is an illustration of the dilution scheme outlined in the Procedure. The dilution assigned to each tube (written below the tube) represents the proportion of original sample inside that tube. For example, if the dilution is 10^{-4}, the proportion of original sample inside the tube would be 1/10,000th of the total volume inside. When 0.1 mL of that solution is transferred to a plate, the volume of sample in the plate is 0.1 mL × 10^{-4} = 10^{-5} mL.

receives a known volume from the mixture in the previous tube and delivers a known volume to the next, typically reducing the cell density to 1/10 or 1/100 at each step. (Greater dilutions in single steps are generally avoided, because they can be accomplished more conveniently and with greater accuracy by simply combining 1/10 or 1/100 dilutions.)

For example, if the original sample in Figure 6.1 contains 1,000,000 cells/mL, following the first transfer the 1/100 dilution in dilution tube 1 would contain 10,000 cells/mL (1,000,000 × 1/100 = 10,000 cells/mL). In the second dilution (tube 2) the 1/100 dilution would reduce it further to 100 cells/mL (10,000 × 1/100 = 100 cells/mL). Because the cell density of the original sample is not known at this time, only the dilutions (without mL units) are recorded on the dilution tubes. By convention, dilutions are expressed in scientific notation. Therefore, a 1/10 dilution is written as 10^{-1} and a 1/100 dilution is written as 10^{-2}.

A small portion of appropriate dilutions (depending on the *estimated* cell density of the original sample) is then spread onto agar plates to produce at least one **countable plate.** A countable plate is one that contains between 30 and 300 colonies (Fig. 6.2). A count lower than 30 colonies is considered statistically unreliable and greater than 300 is typically too many to be viewed as individual colonies.

In examining the procedural diagram (Fig. 6.1), you can see that the first transfer in the series is a simple dilution, but that all successive transfers are compound dilutions. Both types of dilutions can be calculated using the following formula,

$$V_1D_1 = V_2D_2$$

where V_1 and D_1 are the volume and dilution of the concentrated broth, respectively, while V_2 and D_2 are the volume and dilution of the completed dilution. Undiluted samples are always expressed as 1. Therefore, to calculate the dilution of a 1 mL sample transferred to 9 mL of diluent, the permuted formula would be used as follows.

$$D_2 = \frac{V_1D_1}{V_2} = \frac{1.0\text{ mL} \times 1}{10\text{ mL}} = \frac{1}{10} = 10^{-1}$$

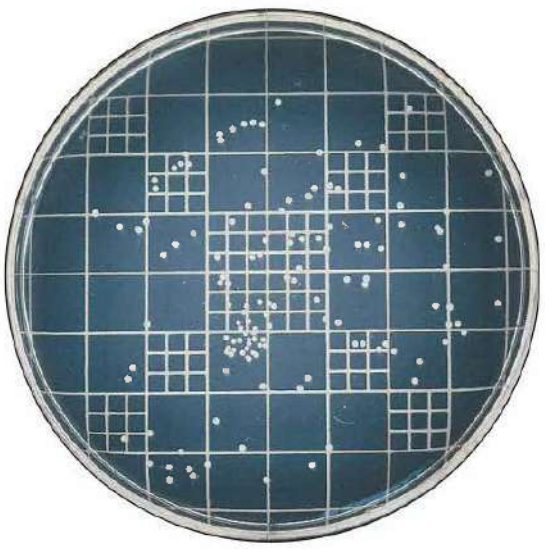
As mentioned above, compound dilutions are calculated using the same formula. However, because D_1 in compound dilutions no longer represents undiluted sample, but rather a fraction of the original density, it must be represented as something less than 1 (e.g., 10^{-1}, 10^{-2}, etc.) For example, if 1 mL of the 10^{-1} dilution from the last example were transferred to 9 mL of diluent, it would become a 10^{-2} dilution as follows.[1]

$$D_2 = \frac{V_1D_1}{V_2} = \frac{1.0\text{ mL} \times 10^{-1}}{10\text{ mL}} = 10^{-1} \times 10^{-1} = 10^{-2}$$

Spreading a known volume of this dilution onto an agar plate and counting the colonies that develop would give you all the information you need to calculate the original cell density (OCD). Below is the basic formula for this calculation.

$$OCD = \frac{CFU}{DxV}$$

CFU (colony forming units) is actually the number of colonies that develop on the plate. CFU is the preferred term because colonies could develop from single cells or from groups of cells, depending on the typical cellular arrangement of the organism. D is the dilution as written on the dilution tube from which the inoculum comes. V is the volume transferred to the plate. (***Note:*** The volume is included in the formula because densities are expressed in CFU/mL, therefore a 0.1 mL inoculation [which would contain 1/10th as many cells as 1 mL] must be accounted for.)



6.2 Countable Plate ◆ A countable plate has between 30 and 300 colonies. Therefore, this plate with approximately 130 colonies is countable and can be used to calculate cell density in the original sample. Plates with fewer than 30 colonies are TFTC ("too few to count"). Plates with more than 300 colonies are TNTC ("too numerous to count").

[1] Permutations of this formula work with all necessary dilution calculations. For calculations involving unconventional volumes or dilutions, the formula is essential, but for simple tenfold or hundredfold dilutions like the ones described in this exercise, the final compounded dilution in a series can be calculated simply by multiplying each of the simple dilutions by each other. For example, a series of three 10^{-1} dilutions would yield a final dilution of 10^{-3} ($10^{-1} \times 10^{-1} \times 10^{-1} = 10^{-3}$). Three 10^{-2} dilutions would yield a final dilution of 10^{-6} ($10^{-2} \times 10^{-2} \times 10^{-2} = 10^{-6}$). We encourage you to use whatever method is best for you. In time you will be doing the calculations in your head.

As you can see in the formula, the volume of *original sample* being transferred to a plate is the product of the *volume transferred* and the *dilution* of the tube from which it came. Therefore 0.1 mL transferred from a 10^{-2} dilution contains only 10^{-3} mL of the original sample (0.1 mL × 10^{-2}). The convention among microbiologists is to condense D and V in the formula into "Original sample volume"[2] (expressed in mL). The formula thus becomes,

$$\text{OCD} = \frac{\text{CFU}}{\text{Original sample volume}}$$

The sample volume is written on the plate at the time of inoculation. Following a period of incubation, the plates are examined, colonies are counted on the countable plates, and calculation is a simple division problem. Suppose, for example, you counted 37 colonies on a plate inoculated a plate with 0.1 mL of a 10^{-5} dilution. Knowing that this plate now contains 10^{-6} mL of original sample, calculation would be as follows.

$$\text{OCD} = \frac{\text{CFU}}{\text{Sample volume}} = \frac{37\ \text{CFU}}{10^{-6}\ \text{mL}} = 3.7 \times 10^{7}\ \text{CFU/mL}$$

Application

The viable count is one method of determining the density of a microbial population. It provides an estimate of actual *living* cells in the sample.

In This Exercise

You will perform a dilution series and determine the population density of a broth culture of *Escherichia coli*. You will inoculate the plates using the spread plate technique, as illustrated in Exercise 1-5. As described in Figure 1.42, the inocula from the dilution tubes will be evenly dispersed over the agar surface with a bent glass rod. You will be sterilizing the glass rod between inoculations by immersing it in alcohol and igniting it. Be careful to organize your work area properly and **at all times keep the flame away from the alcohol jar. Should a fire start in the jar, extinguish it by replacing the lid on the jar.**

✓ Materials

Per Student Group

- ☐ Sterile 0.1 mL, 1.0 mL, and 10.0 mL pipettes
- ☐ Mechanical pipettor
- ☐ Five sterile dilution tubes with caps
- ☐ Flask of sterile normal saline
- ☐ Eight nutrient agar plates
- ☐ Screw-cap jar (and its lid) containing ethanol and a bent glass rod
- ☐ Hand tally counter
- ☐ Colony counter
- ☐ 24-hour broth culture of *Escherichia coli* (this culture will have between 2 × 10^{7} and 2 × 10^{10} CFU/mL)

Procedure

Refer to the procedural diagram in Figure 6.1 as needed. Appendix F provides an alternative procedure using digital micropipettes and µL volumes.

Lab One

1. Obtain eight plates, organize them into four pairs, and label them A_1, A_2, B_1, B_2, etc.
2. Obtain five dilution tubes, and label them 1–5, respectively. Make sure they remain covered until needed.
3. Aseptically add 9.9 mL sterile water to dilution tubes 1 and 2. Cover when finished. Aseptically add 9.0 mL sterile water to dilution tubes 3, 4, and 5. Cover when finished.
4. Mix the broth culture, and aseptically transfer 0.1 mL to dilution tube 1. Mix well. This is a 10^{-2} dilution.
5. Aseptically transfer 0.1 mL from dilution tube 1 to dilution tube 2; mix well. This is a 10^{-4} dilution.
6. Aseptically transfer 1.0 mL from dilution tube 2 to dilution tube 3; mix well. This is a 10^{-5} dilution.
7. Aseptically transfer 1.0 mL from dilution tube 3 to dilution tube 4; mix well. This is a 10^{-6} dilution.
8. Aseptically transfer 1.0 mL from dilution tube 4 to dilution tube 5; mix well. This is a 10^{-7} dilution.
9. Aseptically transfer 0.1 mL from dilution tube 2 to plate A_1. Using the spread plate technique, disperse the sample evenly over the entire surface of the agar. Repeat the procedure with plate A_2, and label both plates "10^{-5} mL original sample," or simply "10^{-5}."
10. Following the same procedure, transfer 0.1 mL volumes from dilution tubes 3, 4, and 5 to plates B, C, and D, respectively. Label the plates accordingly. Allow the inocula to soak into the agar for a few minutes before continuing.
11. Invert the plates and incubate at 35°C for 24 to 48 hours.

[2] Some microbiologists refer to this as the "plate dilution." We prefer to use OSV in this introduction because it emphasizes what is really happening; that is, OSV is the volume of the original sample deposited on the plate.

Lab Two

1. After incubation, examine the plates and determine the countable pair—plates with 30 to 300 colonies. Only one pair of plates should be countable. The remainder should be "too few to count" (TFTC) or "too numerous to count" (TNTC).
2. Count the colonies on both plates and calculate the average (Fig. 6.3). Various methods are available for counting. A simple way is to use a hand tally counter and a felt-tip marker. Mark each colony on the plastic base and simultaneously click the counter.[3] Record these in the table provided on the data sheet, page 473. (*Note*: You may have more than one pair of plates that is countable. For the practice, count all plates that have between 30–300 colonies, and try to identify which pair you have the most confidence in. If no plates are in the 30–300 colony range, count the pair that is closest.)
3. Using the formula provided on the data sheet, calculate the density of the original sample and record it in the space provided.

6.3 Counting Bacterial Colonies ◆ Place the plate upside down on the colony counter. Turn on the light and adjust the magnifying glass until **all** the colonies are visible. Using the grid in the background as a guide, count colonies one section at a time. Mark each colony with a felt-tip marker as you record with a hand tally counter.

[3] Other, more sophisticated counting methods are available. One uses an electronic pen that, when touched to the plastic Petri dish below a colony, records a tally. There are also software systems that capture an image of the plate and then colonies are counted by the computer.

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 149 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Koch, Arthur L. Page 254 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

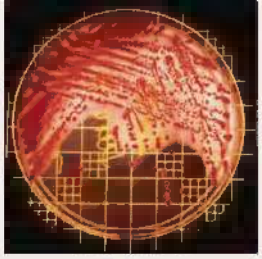
Postgate, J. R. Page 611 in *Methods in Microbiology*, Vol. 1. J. R. Norris and D. W. Ribbons, eds. New York: Academic Press, 1969.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-1

Standard Plate Count (Viable Count)

OBSERVATIONS AND INTERPRETATIONS

1 Enter the number of colonies on each countable plate. Only one pair of plates should be countable, but for practice, record all countable plates anyway. For all plates containing more than 300 colonies, enter TNTC ("too numerous to count"). For plates containing fewer than 30, enter TFTC ("too few to count"). If no plates are countable, use the TFTC plate closest to 30 colonies for practice with the calculations. Make a note of this in step 3 below.

2 Take the average number of colonies from the two (or more) countable plates and record it below.

Plate	A_1	A_2	B_1	B_2	C_1	C_2	D_1	D_2
Colonies counted								
Average # colonies								

3 Calculate the original density in CFU/mL using the following formula:

$$\text{OCD} = \frac{\text{CFU}}{\text{Original sample volume}}$$

Original density of *E. coli* in the broth	

QUESTIONS

When answering the following questions, assume all dilutions are in even powers of ten (unless told otherwise) and that 0.1 mL (100 µL) or 1.0 mL (1,000 µL) volumes were plated.

1 *Suppose your professor handed you a test tube with 2.0 mL of an* E. coli *broth culture in it and told you to make a 10^{-1} dilution of the entire culture. Explain how you would do this. Show your calculations.*

2 *Suppose your professor handed you a test tube with 2.0 mL of an* E. coli *broth culture in it and told you to make a 10^{-2} dilution of the entire culture. Explain how you would do this. Show your calculations.*

3 *How would you produce a 10^{-1} dilution of a 3 mL bacterial sample using the entire 3 mL volume?*

4 *How would you produce a 10^{-2} dilution of a 5 mL bacterial sample using the entire 5 mL volume?*

5 *You have 0.05 mL of an undiluted culture at a density of 3.6×10^6 CFU/mL. You then add 4.95 mL sterile diluent. What is the dilution and what is the final density of cells?*

6 *You have 0.3 mL of an undiluted culture at a density of 4.2×10^7 CFU/mL. You then add 2.7 mL sterile diluent. What is the dilution and what is the final density of cells?*

7 *What is the dilution if 96 mL of diluent is added to 4 mL of a bacterial suspension?*

8 *What is the dilution if 75 mL of diluent is added to 25 mL of a bacterial suspension?*

9 *You were instructed to add 1.0 mL out of 5.0 mL of an undiluted sample to 99 mL of sterile diluent. Instead, you add all 5.0 mL to the 99 mL. What was the intended dilution and what was the actual dilution?*

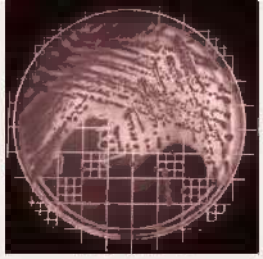
10 *Suppose you were instructed to add 0.2 mL of sample to 9.8 mL of diluent, but instead added 2.0 mL of sample. What was the intended dilution and what was the actual dilution?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-1

(continued)

11 *Plating 1.0 mL of a sample diluted by a factor of 10^{-3} produced 43 colonies. What was the original cell density in the sample?*

_____

12 *Plating 0.1 mL of a sample diluted by a factor of 10^{-3} produced 43 colonies. What was the original cell density in the sample?*

_____

13 *A plate with a sample volume of 10^{-7} mL produced 72 colonies.*

a. *What was the original cell density?*

_____

b. *How many colonies should be on the plate inoculated with a sample volume of 10^{-6} mL?*

_____

c. *How many colonies should be on the plate inoculated with a sample volume of 10^{-8} mL?*

_____

14 *A plate with a sample volume of 10^{-6} mL produced 259 colonies.*

a. *What was the original cell density?*

_____

b. *How many colonies should be on the plate inoculated with a sample volume of 10^{-5} mL?*

_____

c. *How many colonies should be on the plate inoculated with a sample volume of 10^{-7} mL?*

_____

15 *A nutrient agar plate that received 1,000 µL of a bacterial sample diluted by a factor of 10^{-6} had 298 colonies on it after incubation. What was the original cell density?*

_____

16 *You inoculated a nutrient agar plate with 100 µL of a sample diluted by a factor of 10^{-3}. After incubation, you count 58 colonies. What was the original cell density?*

---

17 *A nutrient agar plate labeled 10^{-5} mL produced 154 colonies after incubation.*

a. *What was the cell density in the original sample?*

---

b. *What combination(s) of volumes and dilution factors could have been used to inoculate this plate?*

---

18 *A nutrient agar plate labeled 10^{-7} mL produced 62 colonies after incubation.*

a. *What was the cell density in the original sample?*

---

b. *What combination(s) of volumes and dilution factors could have been used to inoculate this plate?*

---

19 *The original cell density in a sample is 2.79×10^6 CFU/mL. Which sample volume should yield a countable plate? (Express your answer as 10^x mL.)*

---

20 *The original cell density in a sample is 5.1×10^9 CFU/mL. Which sample volume should yield a countable plate? (Express your answer as 10^x mL.)*

---

21 *A sample has a density of 1.37×10^5 CFU/mL.*

a. *What sample volume should yield a countable plate?*

---

b. *Which two dilution tubes could be used to produce this sample volume? How?*

---

22 *A sample has a density of 7.9×10^9 CFU/mL.*

a. *What sample volume should yield a countable plate?*

---

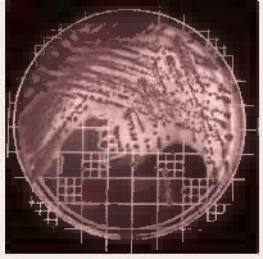
b. *Which two dilution tubes could be used to produce this sample volume? How?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-1

(continued)

23 *You are told that a sample has between 2.5×10^3 and 2.5×10^5 cells/mL. Devise a complete but efficient (that is, no extra plates!) dilution scheme that will ensure getting a countable plate.*

24 *A sample has between 3.3×10^4 and 3.3×10^6 CFU/mL. Devise a complete but efficient (that is, no extra plates!) dilution scheme that will ensure getting a countable plate.*

25 *Two plates received 100 µL from the same dilution tube. The first plate had 293 colonies, whereas the second had 158 colonies. Suggest reasonable sources of error.*

26 *Two parallel dilution series were made from the same original sample. The plates with sample volumes of 10^{-5} mL from each dilution series yielded 144 and 93 colonies. Suggest reasonable sources of error.*

EXERCISE
6-2

Urine Culture

Theory

Urine culture is a semiquantitative CFU counting method that quickly produces countable plates without a serial dilution. The instrument used in this procedure is a volumetric loop, calibrated to hold 0.001 mL or 0.01 mL of sample. Urine culture procedures using volumetric loops are useful in situations where a rapid diagnosis is essential and approximations ($\pm 10^2$ CFU/mL) are sufficient to choose a course of action. Volumetric loops are useful in situations where population density is not likely to exceed 10^5 CFU/mL.

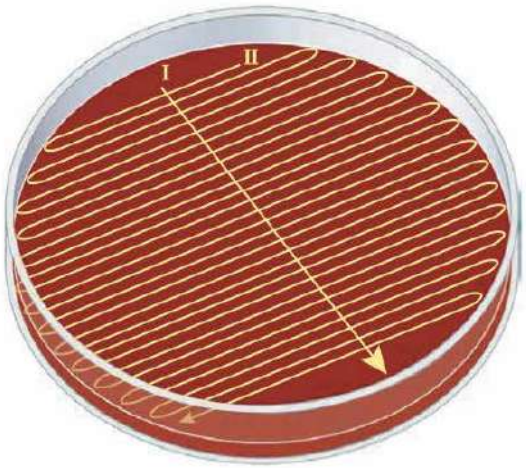
In this standard procedure, a loopful of urine is carefully transferred to a blood agar plate. The initial inoculation is a single streak across the diameter of the agar plate. Then the plate is turned 90° and (without flaming the loop) streaked again, this time across the original line in a zigzag pattern to evenly disperse the bacteria over the entire plate (Figs. 6.4 and 6.5). Following a period of incubation, the resulting colonies are counted and population density, usually referred to as "original cell density," or OCD, is calculated.

OCD is recorded in "colony forming units" per milliliter (CFU/mL), as described in the introduction to this section. CFU/mL is determined by dividing the number of colonies on the plate by the volume of the loop. For example, if 75 colonies are counted on a plate inoculated with a 0.001 mL loop, the calculation would be as follows:

$$\text{OCD} = \frac{\text{CFU}}{\text{Loop volume}}$$

$$\text{OCD} = \frac{75 \text{ CFU}}{0.001 \text{ mL}}$$

$$\text{OCD} = 7.5 \times 10^4 \text{ CFU/mL}$$



6.4 Semiquantitative Streak Method ◆ Streak 1 is a simple streak line across the diameter of the plate. Streak 2 is a tight streak across Streak 1 to cover the entire plate.

6.5 Urine Streak on Sheep Blood Agar ◆ This plate was inoculated with a 0.01 mL volumetric loop. The cell density can be determined by dividing the number of colonies by 0.01.

Application

Urine culture is a common method of detecting and quantifying urinary tract infections. It frequently is combined with media for specific identification of members of *Enterobacteriaceae* (e.g., MacConkey agar) or *Streptococcus* (sheep blood agar).

In This Exercise

You will estimate cell density in a urine sample using a volumetric loop and the above formula. Be sure to hold the loop vertically and transfer slowly.

✓ Materials

Per Student Group

- ☐ One blood agar plate (TSA with 5% sheep blood)
- ☐ One sterile volumetric inoculating loop (either 0.01 mL or 0.001 mL)
- ☐ Fresh urine sample
- ☐ Felt-tip pen
- ☐ Hand tally counter

Procedure

Lab One

1. Thoroughly, but gently, mix the urine with a sterile wooden stick or suitable substitute. Dispose of the stick according to your lab practices (e.g., in a sharps container).
2. Volumetric loops are generally disposable and are in sterile packages. Remove the loop from its package and do not incinerate it.
3. Hold the loop vertically and gently immerse it in the urine sample. Then carefully withdraw it to obtain the correct volume of urine. This loop is designed to fill to capacity in the vertical position. Do not tilt it until you get it in position over the plate.
4. Inoculate the blood agar by making a single streak across the diameter of the plate.
5. Turn the plate 90° and streak the urine across the entire surface of the agar, as shown in Figure 6.4. Dispose of the loop without incineration according to your lab practices.
6. Invert, label, and incubate the plate for 24 hours at 35 ± 2°C.

Lab Two

1. Remove the plate from the incubator and count the colonies using the felt-tip pen and hand tally counter. Touch the plate's base where there is a colony and simultaneously click the counter. Also note any differing colony morphologies, which would indicate possible colonization by more than one species. Enter the data in the table on the data sheet, page 481.
2. Calculate the original cell density of the sample using the formula on the data sheet. If two species are present, calculate each.
3. Enter the cell density(-ies) in the table.

References

Tille, Patricia M. Pages 926–927 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

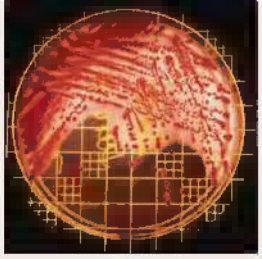
Winn, Washington C., et al. Page 85 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Baltimore: Lippincott Williams & Wilkins, 2006.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-2

Urine Culture

OBSERVATIONS AND INTERPRETATIONS

1 Enter your colony count and loop volume data in the table below. Then calculate the original cell density using the following formula:

$$\text{OCD} = \frac{\text{CFU}}{\text{Loop volume}}$$

Use the extra rows to calculate cell densities of different colony types on your plate or for urine samples of other students. Label these appropriately.

Urine Sample	Colonies Counted	Loop Volume (0.01 mL or 0.001 mL)	Original Cell Density (CFU/mL)

QUESTIONS

1 *The plate pictured in Figure 6.5 was inoculated with a 0.01 mL volumetric loop and contains approximately 75 colonies. What was the original cell density?*

2 *The equation shown in the Theory explanation is used for calculating cell density in urine when using a 0.001 mL calibrated loop. The urine transferred in the loop is not literally diluted, yet its volume is equivalent to a dilution factor.*

a. *What is the dilution factor (based on loop volume) expressed as a fraction?*

b. *What is the dilution factor in scientific notation?*

3 *Calculation of original density in this exercise differs slightly from that offered in Exercise 6-1.*

a. *Compare and contrast the formula used today with that used in Exercise 6-1.*

b. *Could you have used the formula in Exercise 6-1 for today's calculations? Explain.*

4 *Using a volumetric loop is a semiquantitative technique.*

a. *Why is it not quantitative?*

b. *Design a procedure that would make it quantitative.* (**Hint:** *Refer to Exercise 6-1 if necessary.*)

Direct Count (Petroff–Hausser)

EXERCISE 6-3

Theory

Microbial direct counts, like plate counts, take a small portion of a sample and use the data gathered from it to calculate the overall population cell density. This is made possible with a device called a Petroff-Hausser counting chamber. The **Petroff-Hausser counting chamber** is very much like a microscope slide with a 0.02 (2/100) mm-deep chamber, or "well," in the center containing an etched grid (Fig. 6.6). The grid is one square millimeter and consists of 25 large squares, each of which contains 16 small squares, making a total of 400 small squares. Figures 6.7 and 6.8 illustrate the counting grid.

Each small square is 1/20 mm by 1/20 mm, making its area 1/400 mm^2. When the cover glass is put in place 2/100 mm above the grid, the volume above each small square is 1/400 $mm^2 \times 2/100$ mm = 2/40,000 $mm^3 = 5 \times 10^{-5}$ mm^3. One mm^3 equals 10^{-3} cm^3, so 5×10^{-5} $mm^3 = 5 \times 10^{-8}$ cm^3. Because 1 cm^3 = 1 mL, the volume above each small square is 5×10^{-8} mL. This may seem like an extremely small volume—and it is—but the space above each small square is large enough to hold about 50,000 average-size cocci! Fortunately, dilution procedures prevent this scenario from occurring, and cell counting is easily done using the microscope.

As mentioned in the introduction to this section, cell density usually is referred to as "original cell density," or OCD, because most samples must be diluted before attempting a count. OCD is determined by counting the cells found in a predetermined group of small squares and dividing by the number of squares counted multiplied by the dilution[1] and the volume of sample above one small square.

The following is a standard formula for calculating original cell density in a direct count.

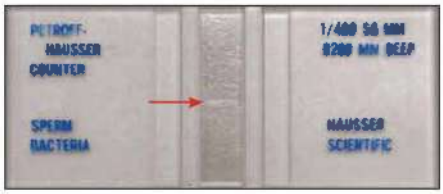
$$\text{OCD} = \frac{\text{Cells counted}}{(\text{Squares})(\text{Dilution})(\text{Volume})}$$

To maintain accuracy, some experts recommend a minimum overall count of 600 cells in one or more

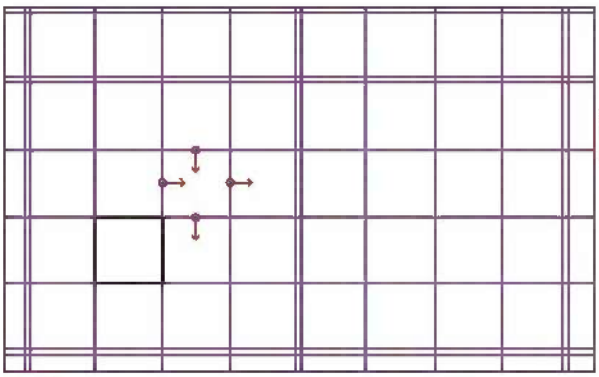
[1] Dilutions are calculated using the following formula.

$$D_2 = \frac{V_1 D_1}{V_2}$$

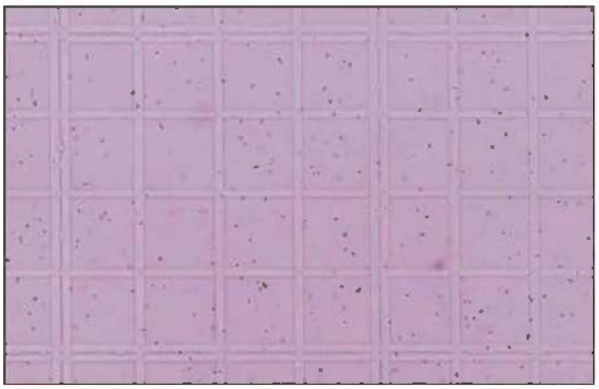
D_2 is the new dilution to be determined. V_1 is the volume of sample being diluted. D_1 is the dilution of the sample before adding diluent (undiluted samples have a dilution factor of 1). V_2 is the new combined volume of sample and diluent after the dilution is completed.



6.6 Petroff-Hausser Counting Chamber ◆ The Petroff-Hausser counting chamber is a precision instrument used for the direct counting of bacterial cells. A bacterial suspension is drawn by capillary action from a pipette into the chamber enclosed by a cover glass. The cells are then counted against the grid of small squares in the center of the chamber.



6.7 Petroff-Hausser Counting Chamber Grid ◆ Shown is a drawing of a portion of the Petroff-Hausser counting chamber grid. The smallest squares are the ones referred to in the formula. The volume above a small square is 5×10^{-8} mL. When cells land on a line, count them with the square below or to the right, as indicated by the cells (violet) and arrows (red).



6.8 Bacterial Cells in a Petroff-Hausser Counting Chamber ◆ This is a 10^{-4} dilution of *Vibrio natriegens* on the grid viewed with the high-dry lens. Try counting cells in several small squares and determine the original cell density.

samples taken from a single population. Optimum density for counting is between 5 and 15 cells per small square.

If, for example, 200 cells from a sample with a dilution factor of 10^{-2} were counted in 16 squares (remembering that the volume above a single small square is 5×10^{-8} mL), the cell density in the original sample would be calculated as follows:

$$\text{OCD} = \frac{\text{Cells counted}}{(\text{Squares})\,(\text{Dilution})\,(\text{Volume})}$$

$$\text{OCD} = \frac{200 \text{ cells}}{(16)(10^{-2})(5 \times 10^{-8}\text{ mL})}$$

$$\text{OCD} = 2.5 \times 10^{10} \text{ cells/mL}$$

Application

The direct count is used to determine bacterial cell density in a sample. One drawback of this method is that living and dead cells are counted.

In This Exercise

6

You will employ the direct count method to estimate cell density in a bacterial sample.

Materials

Per Student Group

- □ Petroff-Hausser (P-H) counting chamber with cover glass
- □ 1 mL pipettes with pipettor
- □ Pasteur pipettes with bulbs or disposable transfer pipettes
- □ Hand counter
- □ Staining agents A and B (Appendix H)
- □ Test tubes
- □ Overnight broth culture of:
  - *Escherichia coli*
  - Container large enough to hold the P-H counting chamber and cover glass, and filled with enough disinfectant to cover the grid
- □ Soft cloth

Procedure

1. Transfer 0.1 mL from the original 24-hour culture tube to a nonsterile test tube.
2. Add 0.4 mL agent A and 0.5 mL agent B and mix well. (This dilution may not be suitable in all situations. There should be 5 to 15 cells per small square for optimal results. Adjust the proportions of the broth culture and agents A and B if necessary to obtain a countable dilution, but try to keep the total solution volume at 1.0 mL for easier calculation of the dilution and cell density.)[2]
3. Handle the Petroff-Hausser counting chamber with care. As of late 2014, each one costs more than $500!
4. Place a cover glass on the Petroff-Hausser counting chamber and add a drop of the mixture at the edge of the cover glass using a nonsterile Pasteur pipette or disposable transfer pipette. Appropriately dispose of the pipette before continuing, being careful not to drip sample while doing so. Capillary action will fill the chamber. Avoid getting sample on the upper surface of the cover glass. It is highly recommended that you clean the slide with disinfectant and alcohol if this happens, rather than risking getting stain on the high-dry lens or letting the sample contaminate other surfaces.
5. Place the counting chamber on the microscope and locate the grid in the center, using the scanning or low-power objective lens. Do not increase the magnification until you have found the grid on one of these lower powers. (***Hint***: Close the iris diaphragm to bring the grid lines into view. Too much light will make them impossible to see.) Increase the magnification, focusing one objective at a time, until you have the cells and the grid in focus with the high-dry lens. Avoid contacting the cover glass with the high-dry lens.
6. Count the number of cells in at least 5 but no more than 16 small squares. Be consistent in your counting of cells that are on a line between squares. By convention, cells on lines belong to the square below or the square to the right.
7. Enter your data in the space provided on the data sheet, page 485, and answer the questions.
8. When you are finished, place the Petroff-Hausser counting chamber in a container of disinfectant, remove the cover glass, and let them soak for 20 minutes. After soaking, rinse with distilled water and gently dry them with a soft cloth.

References

Koch, Arthur L. Page 251 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Postgate, J. R. Page 611 in *Methods in Microbiology*, Vol. 1, J. R. Norris and D. W. Ribbons, eds. New York: Academic Press, Inc., 1969.

[2] If the total solution volume results in something other than 1 mL, you will need to adjust your dilution. For example, if you added 1.0 mL of sample to 0.4 mL agent A and 1.0 mL agent B, your dilution would be 1.0 mL in a total volume of 2.4 mL. In scientific notation, this dilution would be calculated as follows:

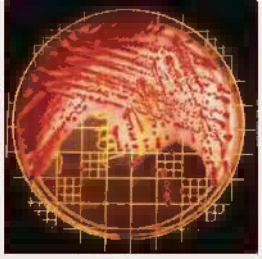
$$\frac{1.0 \text{ mL} \times 10^{0}}{2.4 \text{ mL} \times 10^{0}} = \frac{1.0 \text{ mL}}{2.4 \text{ mL}} \times 10^{0\ -\ 0} = 0.42 = 4.2 \times 10^{-1}$$

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-3

Direct Count (Petroff–Hausser)

DATA AND CALCULATIONS

1 Calculate your dilution using the following formula. For an explanation of dilution, refer to Exercise 6-1.

$$D_2 = \frac{V_1 D_1}{V_2}$$

2 Enter your data in the table.

3 Calculate the original cell density of the *E. coli* culture using the following equation. Record your answer in cells/mL in the table.

$$\text{Original cell density} = \frac{\text{Total cells counted}}{(\text{Squares counted})(\text{Dilution})(5 \times 10^{-8}\ \text{mL})}$$

Total Cells Counted	Squares Counted	Dilution	Calculated Original Cell Density (cells/mL)

QUESTIONS

1 *What are the advantages and disadvantages of the direct count over the plate count technique?*

2 *Suppose that after diluting a sample by 10^{-1} you count 75 cells in 5 small squares of a P-H counting chamber. What is the cell density (in cells/mL) of the original sample?*

3 *You have diluted a sample by 10^{-2} and then count 158 cells in 8 small squares of the P-H counting chamber. What is the cell density (in cells/mL) of the original sample?*

4 *Suppose you had to count a culture that was very turbid and found that the recommended dilution of 0.4 mL stain A and 0.5 mL stain B per 0.1 mL culture (10^{-1} dilution) was not enough. How would you adjust the volumes to make a 10^{-2} dilution? Show your work.*

5 *Suppose you were given a diluted broth to count that already had a 10^{-3} dilution and used the staining procedure outlined in this exercise.*

a. *What would the dilution of the sample be when it reached the counting chamber?*

b. *What would the original cell density be if you counted a total of 112 cells in 16 small squares? Record your answer in cells/mL.*

EXERCISE 6-4

Closed-System Growth

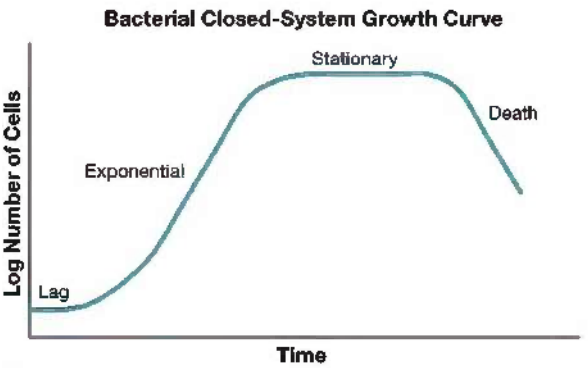
Theory

A closed system—in this exercise, a broth culture in a flask—is one in which no nutrients are added beyond those present in the original medium and no wastes are removed. Bacteria grown in a closed system demonstrate four distinct growth phases: lag phase, exponential phase, stationary phase, and death phase.

The four phases together form a characteristic shape known as a microbial growth curve (Fig. 6.9). **Lag phase**, the first phase, constitutes what might be called an adjustment period. Initially, there is no cell division. It is believed that microorganisms use this time to repair damaged cellular components and synthesize enzymes to begin using the resources of the new environment. The duration of lag phase can be quite variable and depends on many factors. Actively reproducing cells transferred to a medium identical to the one from which they are removed will undergo a short lag period. Cells that are near death, have been refrigerated, or must adjust to a completely different medium will demonstrate a longer lag phase.

Eventually, as cells begin to use resources of the new environment and start reproducing, lag phase gradually gives way to the **exponential** (or **log**) **phase**. This phase is a time of maximum growth that is limited almost exclusively by the organism's reproductive potential. In a closed system, conditions usually are adjusted to be optimal for a specific organism; however, even under the best of conditions, the medium and other physical factors influence the growth rate slightly. Regardless, exponential growth represents the maximum growth rate of that organism under the existing conditions. Exponential growth is characterized by cellular division and doubling of the population size at regular intervals, depending on the organism's generation time. Because not all cells are dividing at exactly the same time, exponential growth is marked by a smooth and dramatic upward sweep of growth, as shown in Figure 6.9.

As nutrients in the medium decrease and toxic waste products increase, the growth rate declines to where the population's death rate is more or less equal to its reproductive rate. This leveling of growth is called the **stationary phase**. Notice that the transition into stationary phase is pretty sudden. This is because toward the end of exponential growth the population is still doubling, but the population size that is doubling is very large, which puts pressure on the system quickly. Stationary phase will last until the nutrients are depleted or the medium becomes toxic to the organism.



6.9 Microbial Closed-System Growth Curve ♦ Note the different phases of population growth produced as a result of no new nutrients being added and no wastes being removed from the closed system.

Death phase, the final phase, is marked by the decline of the organism. As discussed in Exercise 6-6, microbial death is the reverse of microbial growth and is exponential. That is, a fixed proportion of the population will die in a given time (the time is specific to the organism), regardless of population size.

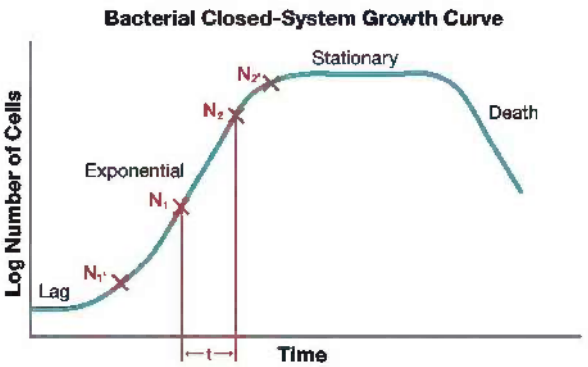
It is customary to plot growth curves on a semilogarithmic graph, where equal increments on the *y*-axis scale represent changes in the exponent. For instance, equal increments on the scale might be 10^1, 10^2, 10^3, and 10^4, rather than a more typical sequence of 5, 10, 15, and 20. This is done for two reasons. One, it allows plotting of data over a large numerical range, and two, a straight line results if the data are produced by a constant exponential function. Conversely (and usefully), seeing a straight line on a log graph tells the reader that the change is constant.

Several measurements are possible in a closed-system growth experiment. These measurements include the duration of each phase, the **mean growth rate constant**, **generation time**, and the organism's **minimum**, **maximum**, and **optimum** (**cardinal**) **temperatures.**

Mean growth rate constant (k) is calculated using the increase in population size between two points in time during exponential growth (Fig. 6.10). The following is the standard formula:

$$k = \frac{\log N_2 - \log N_1}{(0.301)\, t}$$

where N_1 is the population size early in exponential phase, N_2 is the population size later in exponential phase, t is the



6.10 Calculating Growth Rate Constant ◆ When you calculate the growth rate constants for the *V. natriegens* cultures, use two points on the linear part of exponential growth for N_2 and N_1 (or A_2 and A_1). These should be chosen from the data table instead of read off the graph directly. Including data points in the transition between lag and exponential (N_1') and/or exponential and stationary phases (N_2') will affect your calculation by underestimating mean growth rate.

elapsed time between the N_1 and N_2 readings, and 0.301 is the log of 2. Log 2 is used because bacterial reproduction typically occurs by binary fission; therefore, each reproductive cycle results in a doubling of the population size. Calculations of organisms using a different reproductive pattern require the log of a value appropriate to their typical number of offspring.

If actual values for population size are not required, indirect measurements of cell number can be used to calculate *k*. You already know that as the number of cells increases, the turbidity of a culture also increases and absorbs more light. Absorbance can be quantified using a spectrophotometer and absorbance readings taken early and late in the exponential phase (A_1 and A_2) can be substituted in the numerator as follows:

$$k = \frac{\log A_2 - \log A_1}{(0.301)\, t}$$

For example, the mean growth rate constant of an organism whose absorbance readings increase from 0.195 to 0.815 in 2 hours would be:

$$k = \frac{\log 0.815 - \log 0.195}{(0.301)\ 2\ \text{hr}}$$

$$k = \frac{0.62}{0.602\ \text{hr}}$$

$$k = 1.03\ \text{generations/hr}$$

Where *k* is the number of generations per unit time (e.g., generations/hour), *g* is mean generation time—the amount of time required for a population to double—and has units of time/generation (e.g. minutes per generation). Mean generation time is simply the inverse of *k*. It can be easily calculated using the following formula:

$$g = \frac{1}{k}$$

Taking the above example, the generation time of the organism would be:

$$g = \frac{1}{1.03\ \text{generations/hr}}$$

$$g = 0.97\ \text{hr/generation or } 58\ \text{minutes/generation}$$

Application

This exercise is designed to demonstrate the pattern of microbial growth in a closed system, expose you to the methods involved in determining growth, and demonstrate the calculation of mean growth rate and mean generation time.

In This Exercise

You will use a spectrophotometer to measure the growth of *Vibrio natriegens* incubated at as many as six different temperatures. The class will be split into six groups (or as many as is convenient) with each group incubating at a different temperature. Absorbance readings will be taken every 15 minutes. Using the absorbance data collected for your group's culture, you will plot its growth curve either by computer or on the graph paper provided, with absorbance substituting for population size. You then will calculate its mean growth rate constant and generation time. Finally, using the class data, you will plot the mean growth rate constants over the range of temperatures to estimate the minimum, maximum, and optimum growth (cardinal) temperatures for the organism.

✓ Materials

Per Class

- ☐ 20 mL overnight broth culture of *Vibrio natriegens* in brain-heart infusion (BHI) + 2% NaCl
- ☐ Pipette disposal setup

One of Each per Student Group

- ☐ Six water baths set at 22°C, 25°C, 31°C, 37°C, 40°C, and 43°C
- ☐ Thermometers (to monitor actual temperatures in the ice and water baths)
- ☐ Six sterile side-arm flasks containing 47 mL of sterile BHI broth + 2% NaCl
- ☐ Six sterile side-arm flasks containing 50 mL BHI + 2% NaCl to be used as blanks for the spectrophotometers

- ☐ As many as six spectrophotometers
- ☐ Lab tissues
- ☐ Sterile 5 mL pipettes and mechanical pipettor or micropipettes (10–100 µl and 100–1,000 µl) with sterile tips
- ☐ Bunsen burner

Medium Recipe

Brain-Heart Infusion Agar

• Infusion from calf brains	200 g
• Infusion from beef hearts	250 g
• Peptone	10 g
• Dextrose	2 g
• Sodium chloride	5 g
• Disodium phosphate	2.5 g
• Distilled or deionized water	1.0 L
pH 7.2–7.6 at 25°C	

Procedure

Preliminary Notes

- Your instructor will determine which temperatures to use in the experiment if all six are not used. We recommend at least 25°C, 37°C, and 43°C.
- These instructions presume a working knowledge of how to use the spectrophotometer. If you need help, refer to Appendix E.
- It is best if the water baths are at their respective temperatures and the spectrophotometers with lab tissues are set up near them prior to class.
- **It is important that all groups start at the same time.** We recommend having two representatives from each group bring their flask to a designated place in the lab where the *V. natriegens* overnight culture and pipetting materials (including disposal) are located. The table should be covered with disinfectant-soaked towels. Have each group withdraw their 3 mL sample and hold it over the towels without touching the pipette tip. When the last group has withdrawn its sample, all groups should inoculate their flasks simultaneously. If dripping is a problem with glass pipettes, then have each group inoculate their broths immediately and minimize the time between the first and last group as much as possible.
- While inoculation of the flasks is taking place, remaining group members should be calibrating the spectrophotometer with the blank.

Specific Instructions

1. Obtain the side-arm flasks containing 47 mL and 50 mL of BHI broth and locate your assigned water bath and spectrophotometer.
2. Label your 47 mL growth flask with its incubation temperature. Label the 50 mL flask "negative control." Place both into the appropriate water bath and allow their temperatures to equilibrate (10–15 minutes).
3. If this has not already been done, turn on the spectrophotometer and let it warm up for a few minutes.
4. If the spectrophotometer is digital, set it to "absorbance." Set the wavelength to 650 nm and make sure the red filter is in place.
5. When all groups are ready to start (all groups *must* start at the same time), split your group into two.
   a. Two students should remove their 50 mL flask from the water bath, dry it, and take it to the table with the *V. natriegens* overnight culture. The table should be covered with disinfectant-soaked towels in case culture drips from the pipettes. (*V. natriegens* is a BSL-1 organism and is not known to be harmful to humans. Nevertheless, be careful with it.)
   b. Meanwhile, remaining group members should "blank" the spectrophotometer with the control flask.
6. When instructed to do so, obtain a 5 mL pipette and insert it into the mechanical pipettor. Do not touch the tip. (If using a digital pipettor, attach the tip.) Mix the *V. natriegens* culture.
7. When instructed to do so, withdraw 3 mL with the pipette and hold it over the towel. When the last group has drawn their 3 mL of culture, inoculate your flask simultaneously with the other groups. This marks the beginning of the experiment (time zero = t_0) Dispose of the pipette or tip according to your lab protocols.
8. Return with your flask to the spectrophotometer and take the first reading. The spectrophotometer should already be calibrated and ready to use.
   a. Gently swirl the contents in the flask, wipe it dry (if necessary), and make sure the side arm is clean.
   b. Carefully insert the side arm into the sample port and take the reading (Fig. 6.11). Outside light will affect your readings and the lid to the sample port cannot be closed, so use your fingers or a paper towel to cover the opening.

6.11 Side-arm Flask in the Spectrophotometer ◆ Insert and remove the flask carefully to avoid spilling the broth. Place the flask in the spectrophotometer the same direction each time to avoid inconsistent readings caused by aberrations in the glass. Cover the port with your fingers to reduce light entering the machine.

6

c Be careful not to insert or remove the flask too quickly, because it is very easy to spill from the side-arm flask when it is tipped for reading. Further, if it is pulled out on an angle, you risk breaking the side arm and spilling the culture—which creates a nightmare of a mess to clean up and also stops your experiment.

d Even though it may have been a minute or more since inoculation, this is your t_0 absorbance reading. Record the absorbance next to your temperature under t_0 in Table A on the data sheet, page 491.

e Remove the growth flask carefully, swirl it to mix the organisms, and return it and the control flask to the water bath until the next reading.

9 Repeat step 8 every 15 minutes (t_{15}, t_{30}, t_{45}, etc.), taking absorbance readings and recording them in Table A until you have completed and recorded the t_{240} reading (or however long your lab meets). Make sure to place the flask in the spectrophotometer the same direction each time (to compensate for irregularities in the glass) and to cover the port with your fingers. We recommend that the spectrophotometer be calibrated about 2 minutes before each reading.

10 Monitor the temperature in your water/ice bath frequently. It is more important to have a constant temperature than to have the exact assigned temperature. Record your *actual* temperature in Tables A, B, and C on the data sheet.

References

Collins, C. H., Patricia M. Lyne, J. M. Grange. Page 149 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Gerhardt, Philipp, et al. Chapter 11 in *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology, 1994.

Koch, Arthur L. Page 251 in *Methods for General and Molecular Bacteriology*, Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Postgate, J. R. Page 611 in *Methods in Microbiology*, Vol. 1. J. R. Norris and D. W. Ribbon, eds. New York: Academic Press, 1969.

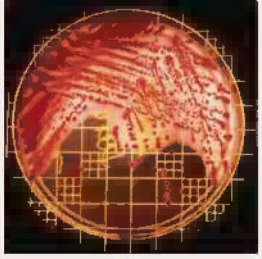
Prescott, Lansing M., John P. Harley, and Donald A. Klein. Page 114 in *Microbiology*, 4th ed. Boston: WCB/McGraw-Hill, 1999.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-4

Closed-System Growth

DATA AND CALCULATIONS

1 Enter the absorbance values for all groups in Table A.

2 On a computer or the graph paper provided in this data sheet, plot your absorbance values versus time. This will produce a growth curve including growth phases as far as the stationary phase. (***Note:*** Some cultures may not even reach the stationary phase. Record what you get!). Also plot the growth curves of the other five samples on the same set of axes.

3 From your graph, determine the approximate length of time spent in the various growth stages for all samples, and enter those values in Table B. These should add up to 240 minutes (or however long your lab ran the experiment).

4 From your graph, determine the approximate absorbance for the lag phase and the stationary phase of each culture, and enter those in Table B.

5 Calculate the mean growth rate constant for each sample. Do this by choosing two points clearly on the linear part of exponential growth. These are A_1 and A_2 (Fig. 6.10). Determine the absorbance values of each, and the time in minutes (t) between the two points. Substitute your values in the equation and solve for k. Enter your results in Table C.

$$k = \frac{\log A_2 - \log A_1}{(0.301)t}$$

6 On a computer or the graph paper provided, plot the mean growth rate (from Table C) versus temperature of the different samples. Use different colors or shapes for the data points for each temperature.

7 Calculate generation times of the different samples. Enter your results in Table C.

Table A—Absorbance Readings

Temp (°C)	t_0	t_{15}	t_{30}	t_{45}	t_{60}	t_{75}	t_{90}	t_{105}	t_{120}	t_{135}	t_{150}	t_{165}	t_{180}	t_{195}	t_{210}	t_{225}	t_{240}
22																	
25																	
31																	
37																	
40																	
43																	

Table B – Growth Phase Analysis					
Temp (°C)	Lag Phase Duration (minutes)	Exponential Phase Duration (minutes)	Stationary Phase Duration (minutes)	Magnitude of Lag Phase (absorbance)	Magnitude of Stationary Phase (absorbance)
22					
25					
31					
37					
40					
43					

6

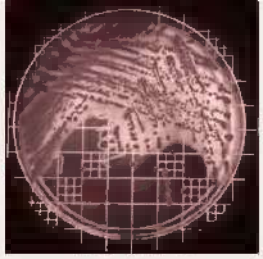
Table C – Calculations (Include Appropriate Units)					
Temp (°C)	A_2 / $\log A_2$	A_1 / $\log A_1$	t	k	g
22					
25					
31					
37					
40					
43					

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-4

(continued)

QUESTIONS

1 *Examine the microbial growth curve in Figure 6.9. In what ways would you expect it to be different from a growth curve of an organism living in a natural environment?*

2 *What environmental factor(s) prevented your culture from being a completely closed system?*

3 *Aside from temperature, what factors in today's experiment likely had an effect on the growth? What factors likely kept the organism from realizing true exponential growth?*

4 *If an organism has a mean generation time of 22 minutes, what is its mean growth rate?*

5 *If an organism has a mean generation time of 47 minutes, what is its mean growth rate?*

6 *If an organism has a mean growth rate constant of 1.7 generations per hour, what is its generation time?*

7 *If an organism has a mean growth rate constant of 0.012 generations per minute, what is its generation time?*

8 *How would you calculate mean growth rate of an organism that quadrupled every generation?*

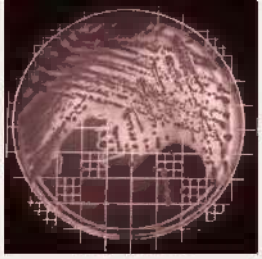
9 *Compare your growth rate vs. temperature graph with those shown in Figure 2.47. How would* Vibrio natriegens *be classified?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-4

(continued)

Plaque Assay of Virus Titer

EXERCISE 6-5

Theory

Viruses that attack bacteria are called **bacteriophages**, or simply **phages** (Fig. 6.12). Some viruses attach to the bacterial cell wall and inject viral DNA into the bacterial cytoplasm. The viral **genome** then commands the **host cell** to produce more viral DNA and viral proteins, which are used for the assembly of more phages. Once assembly is complete, the cell lyses and releases the phages, which then attack other bacterial cells and begin the replicative cycle all over again. This process, called the **lytic cycle**, is shown in Figure 6.13.

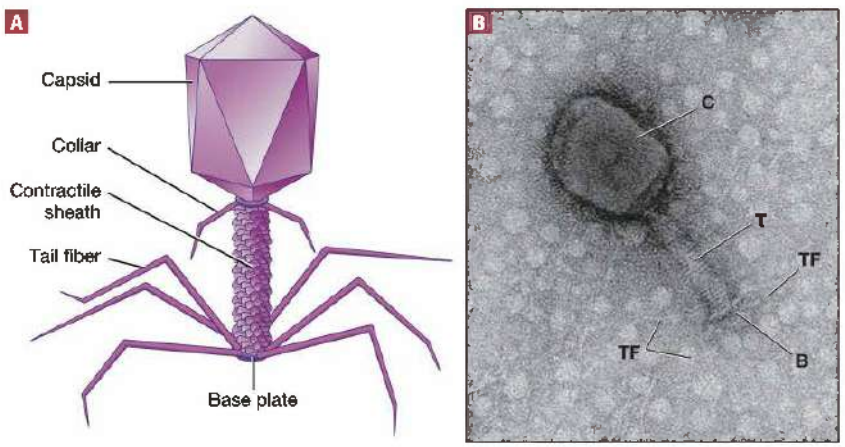
Lysis of bacterial cells growing in a lawn on an agar plate produces a clearing that can be viewed with the naked eye. These clearings are called **plaques**. Plaque assay uses this phenomenon as a means of calculating the phage concentration in a given sample. When a sample of bacteriophage (generally diluted by means of a serial dilution) is added to a plate inoculated with enough bacterial host to produce a lawn of growth, the number of plaques formed can be used to calculate the original phage **titer**, or density. Refer to the procedural diagram in Figure 6.14.

Plaque assay technique is similar to the standard plate count, in that it employs a serial dilution to produce countable plates needed for later calculations. (*Note*: Refer to Exercise 6-1, Standard Plate Count, as needed for a description of serial dilutions, dilution factors, and calculations.) One key difference is that the plaque assay is done using the **pour-plate technique**, in which bacterial cells and viruses are first added to molten agar and then poured into the plate.

In this procedure, diluted phage is added directly to a small amount of *E. coli* culture and allowed a 10-minute (±5 minutes) **preadsorption period** to attach to the bacterial cells. Then this phage–host mixture is added to a tube of **soft agar**, mixed, and poured onto prepared nutrient agar plates as an **agar overlay**. The consistency of the solidified soft agar is sufficient to immobilize the bacteria while allowing the smaller bacteriophages to diffuse short distances and infect surrounding cells. During incubation, the phage host produces a lawn of growth on the plate in which plaques appear where contiguous cells have been lysed by the virus (Fig. 6.15).

The procedure for counting plaques is the same as that for the standard plate count. To be statistically reliable, countable plates must have between 30 and 300 plaques. Calculating phage titer (original phage density) uses the same formula as other plate counts except that PFU (plaque forming unit) instead of CFU (colony forming unit) becomes the numerator in the equation. Phage titer, therefore, is expressed in PFU/mL and the formula is written as follows:

$$\text{Phage titer} = \frac{\text{PFU}}{\text{Volume plated} \times \text{Dilution}}$$



6.12 T4 Coliphage ◆ (A) As shown in this artist's rendition, bacteriophages frequently have a complex structure that includes the capsid containing the genome and a tail with many parts. T4 phage has an icosahedral (20 triangular faces) capsid. (B) This is a negative stain of one T4 phage particle. Shown are the capsid (C), tail (T), base plate (B), and tail fibers (TF). The length of this phage from base plate to tip of capsid is approximately 180 nm (0.18 µm).
(Photo taken by author at the San Diego State University Electron Microscope Facility.)

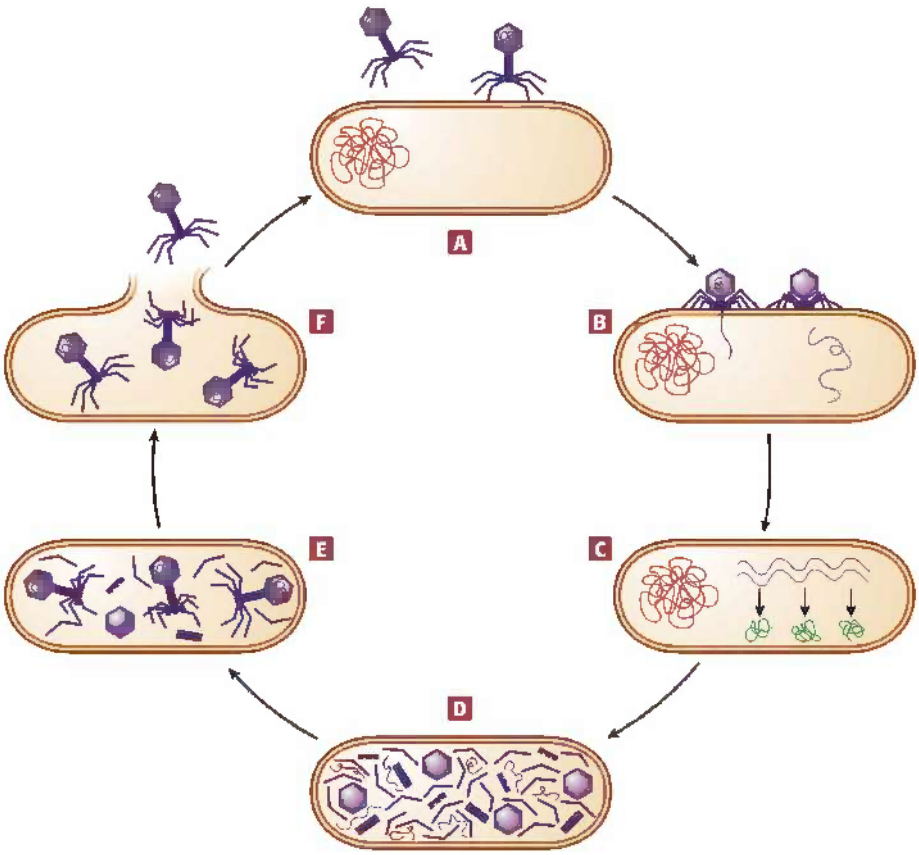
As with the standard plate count (Exercise 6-1), it is customary to condense *volume plated* and *dilution* into *original sample volume*. The formula then becomes:

$$\text{Phage titer} = \frac{\text{PFU}}{\text{Original sample volume}}$$

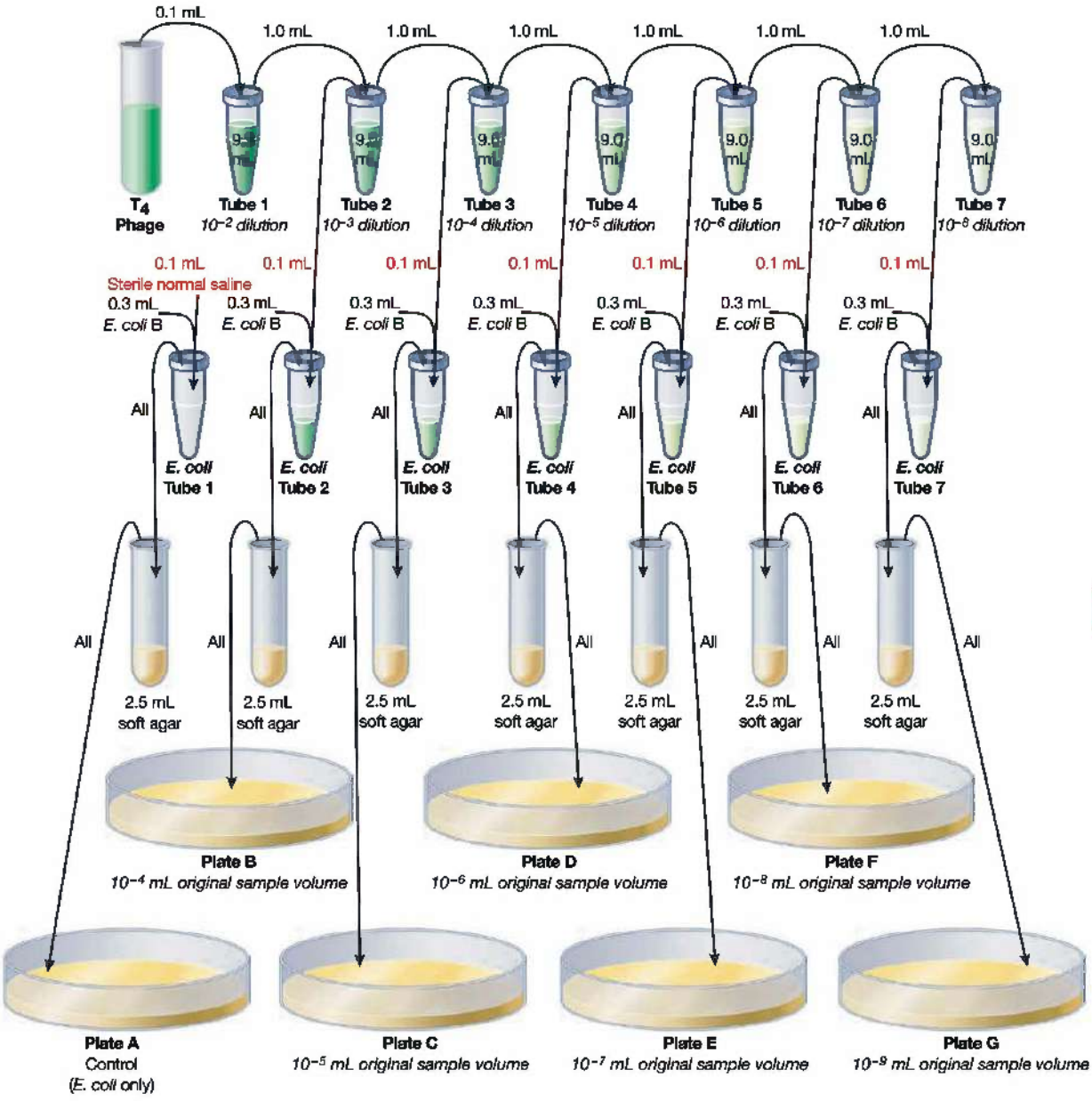
The original sample volume is written on the plate at the time of inoculation. Following a period of incubation, the plates are examined, plaques are counted on the countable plates, and calculation is a simple division problem.

Suppose, for example, you inoculated a plate with 0.1 mL of a 10^{-4} dilution. (Remember, you are calculating the *phage density*; the *E. coli* has nothing to do with the calculations.) This plate now contains 10^{-5} mL of original phage sample. If you subsequently counted 115 plaques on the plate, calculation would be as follows:

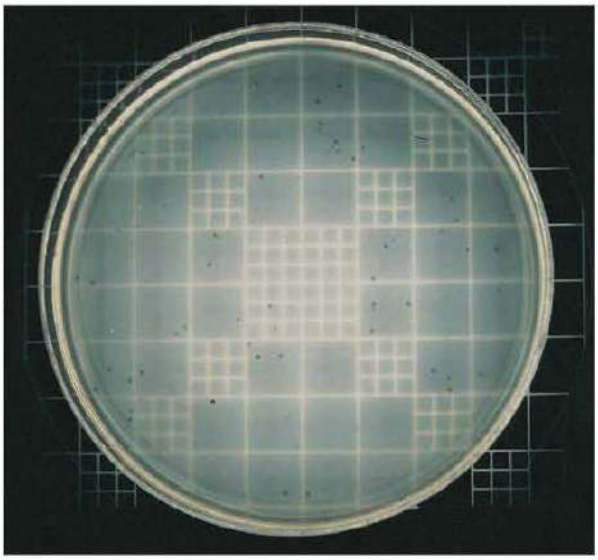
$$\text{Original phage density} = \frac{1.15 \times 10^2 \text{ PFU}}{10^{-5} \text{ mL}} = 1.15 \times 10^7 \text{ PFU/mL}$$



6.13 T4 Phage Lytic Cycle ◆ Shown is a simplified diagram of the T4 coliphage's replicative cycle. In **A**, an infective T4 phage (left) is approaching its host, *Escherichia coli*. On the right, the same phage is shown attached by its legs to specific receptors on *E. coli*. This is the **attachment** phase. The *E. coli* chromosome is the red tangled line at the left of the cell. On the left of **B**, T4 is in the process of transferring its DNA (purple line) into *E. coli*. This is the **penetration** phase. On the right, the entire phage genome is in the host. Removal of viral genome from its capsid is **uncoating**. In **C**, phage DNA is in the process of being transcribed into mRNA (parallel red line), which is then translated into phage proteins (squiggly green lines). This is the **synthesis** phase and is performed by *E. coli* under the direction of the phage DNA. Simultaneously, phage DNA is being replicated, but this is not shown. Synthesis leads to **assembly** of the phage progeny, shown in **D** and **E**, where the capsid subunits come together to form the capsid into which the genome is inserted, and the tail with all its diverse parts comes together and attaches to the capsid. Note that host DNA has been degraded, but remains as short, red lines. (This is important because occasionally *E. coli* DNA is put into a viral capsid and is transferred to a new *E. coli* host, providing it with new genes in a process called **generalized transduction**). In **F**, the fully assembled phages are **released** as the cells bursts. At 37°C, this entire cycle can take as little as 25 minutes and release a few hundred phage progeny, each of which can repeat the process!



6.14 Procedural Diagram for Plaque Assay ◆ This is an illustration of the dilution scheme outlined in the Procedure. The dilution assigned to each tube (written below the tube in the first row) represents the proportion of original sample inside that tube. For example, if the dilution is 10^{-4}, the proportion of original sample inside the tube would be 1/10,000th of the total volume inside. When 0.1 mL of that solution is transferred to a plate, the volume of sample in the plate is 0.1 mL × 10^{-4} or 10^{-5} mL. (***Note:*** Do not be confused by the addition of *E. coli*; it does not affect the phage volume going to the plate.)



6.15 Countable Plate ◆ This plaque assay plate has between 30 and 300 plaques; therefore, it is countable.

6

Application

This technique is used to determine the concentration of viral particles in a sample. Samples taken over a period of time can be used to construct a viral growth curve.

In This Exercise

You will be estimating the density (titer) of a T4 coliphage sample using a strain of *Escherichia coli* (*E. coli B*) as the host organism.

✓ Materials

Per Class

- ☐ 50°C hot-water bath containing tubes of liquid soft agar (seven tubes per group)

Per Student Group

- ☐ Sterile 0.1 mL, 1.0 mL, and 10.0 mL pipettes
- ☐ Seven sterile test tubes in a test tube rack
- ☐ Seven sterile capped microtubes in a microtube holder (rack)
- ☐ Seven tryptic soy agar or nutrient agar plates
- ☐ Seven tubes containing 2.5 mL soft agar
- ☐ 100 mL sterile normal saline in a flask
- ☐ Seven sterile transfer pipettes
- ☐ T4 coliphage
- ☐ 24-hour broth culture of *Escherichia coli B* (T-series phage host)

Medium Recipe

Soft Agar

• Beef extract	3.0 g
• Peptone	5.0 g
• Sodium chloride	5.0 g
• Tryptone	2.5 g
• Yeast extract	2.5 g
• Agar	7.0 g
• Distilled or deionized water	1.0 L

Procedure

Refer to the procedural diagram in Figure 6.14 as needed. Appendix F provides an alternative procedure using digital micropipettes and µL volumes.

Lab One

1. Obtain all materials except for the soft agar tubes. To keep the agar tubes liquefied, leave them in the water bath and take them out one at a time as needed.
2. Label the seven sterile test tubes 1 through 7. These will be used for phage dilution (row one in Fig. 6.14). Label the seven microtubes *E. coli* 1–7. These will be used to mix the diluted phage with *E. coli B* (row two in Fig. 6.14). Place all tubes in an appropriate rack in numerical order.
3. Label the TSA (or nutrient agar) plates A through G. Place them in the 35°C incubator to warm them. Take them out one at a time as needed. This will keep the soft agar (to be added at step 14) from solidifying too quickly and result in a smoother agar surface.
4. Aseptically transfer 9.9 mL sterile normal saline to dilution tube 1.
5. Aseptically transfer 9.0 mL sterile normal saline to dilution tubes 2–7.
6. Mix the *E. coli* culture and aseptically transfer 0.3 mL into each of the *E. coli* tubes.
7. Mix the T4 suspension, and aseptically transfer 0.1 mL to dilution tube 1. Mix well. This is a 10^{-2} dilution.
8. Aseptically transfer 1.0 mL from dilution tube 1 to dilution tube 2. Mix well. This is a 10^{-3} dilution.
9. Aseptically transfer 1.0 mL from dilution tube 2 to dilution tube 3. Mix well. This is a 10^{-4} dilution.
10. Continue in this manner through dilution tube 7. The dilution of tube 7 should be 10^{-8}.

11 Aseptically transfer 0.1 mL sterile normal saline to *E. coli* tube 1. This will be mixed with 2.5 mL soft agar and used to inoculate a control plate.

12 Aseptically transfer 0.1 mL from dilution tube 2 to its companion *E. coli* tube. Repeat this procedure with the remaining five tubes.

13 This is the beginning of the preadsorption period. Let all seven tubes stand undisturbed for 10 ± 5 minutes.

14 Remove one soft agar tube from the water bath and add the entire contents of *E. coli* tube 1 with a sterile transfer pipette. Mix well with the pipette and immediately transfer it onto plate A. Gently tilt the plate back and forth until the soft agar mixture is spread evenly across the solid medium. Label the plate "Control."

15 Remove a second soft agar tube from the water bath and add the entire contents of *E. coli* tube 2 with a sterile transfer pipette. Mix well with the pipette and immediately transfer it onto plate B. Tilt back and forth to cover the agar, and label it 10^{-4} mL (this is the phage sample volume).

16 Repeat this procedure with dilutions 10^{-4} through 10^{-8} and plates C through G. Label the plates with the appropriate sample volume.

17 Allow the agar to solidify completely.

18 Invert the plates and incubate aerobically at 35 ± 2°C for 24 to 48 hours.

Lab Two

1 After incubation, examine the control plate for growth and the absence of plaques.

2 Examine the remainder of your plates and determine which one is countable (30 to 300 plaques). Count the plaques and record the number in the table provided on the data sheet, page 503. Record all others as either TNTC (too numerous to count) or TFTC (too few to count).

3 Using the sample volume on the countable plate and the formula provided on the data sheet, calculate the original phage titer. Record your results on the data sheet.

References

Collins, C. H., Patricia M. Lyne, J. M. Grange. Page 149 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

DIFCO Laboratories. Page 619 in *DIFCO Manual*, 10th ed. Detroit: DIFCO Laboratories, 1984.

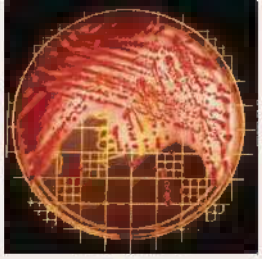
Province, David L. and Roy Curtiss III. Page 328 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-5

Plaque Assay of Virus Titer

OBSERVATIONS AND INTERPRETATIONS

1 Enter the number of plaques counted on the countable plate. Only one plate should be countable. If there are more than 300 plaques, enter TNTC. If fewer than 30 plaques, enter TFTC.

Plate	A	B	C	D	E	F	G
Plaques counted							
Sample volume							

2 Calculate the original density using the following formula, and enter the result below. Record your answer in PFU/mL.

$$\text{Phage titer} = \frac{\text{PFU}}{\text{Original sample volume}}$$

If you don't have any countable plates, use the TFTC plate closest to 30 in your calculation for practice. Make a note of this in your results.

Phage titer	

QUESTIONS

1 *In this exercise, there must be enough bacteria inoculated to produce a lawn of growth. Why is that important?*

2 *Consider the control plate (Plate A) inoculated with only* E. coli B.

a. *Is it an example of a positive or a negative control?*

b. *What purpose did it serve? Be descriptive in your answer.*

3 *Consider the preadsorption step.*

a. *How might the results be altered if you had skipped the preadsorption phase?*

b. *How might the results be altered if you allowed it to run for 35 minutes?*

4 *Consider the water bath.*

a. *Why was it set at 50°C?*

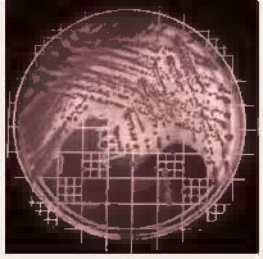
b. *What might be some consequences of raising its temperature? Of lowering its temperature?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-5

(continued)

5 *Consider the soft agar.*

a. *Why was soft agar used for the agar overlay?*

b. *What would you expect to see if standard nutrient agar or trypticase soy agar had been used instead?* (**Hint:** *It might be helpful to look at the recipes for these in Appendix H.*)

6 *For each data set, calculate the original phage titer.*

a. *Phage dilution tube:* 10^{-4}; E. coli B: *300 µL; soft agar: 2.5 mL; countable plate: 37 plaques.*

b. *Phage dilution tube:* 10^{-8}; E. coli B: *300 µL; soft agar: 2.5 mL; countable plate: 216 plaques.*

c. *Phage dilution tube:* 10^{-5}; E. coli B: *300 µL; soft agar: 2.5 mL; countable plate: 37 plaques.*

d. *Phage dilution tube:* 10^{-5}; E. coli B: *500 µL; soft agar: 2.5 mL; countable plate: 37 plaques.*

e. *Phage dilution tube:* 10^{-5}; E. coli B: *300 µL; soft agar: 2.0 mL; countable plate: 37 plaques.*

7 *Examine Figure 6.15. Suppose the OSV on this plate was* 10^{-6} *mL. What was the original phage titer?*

Thermal Death Time Versus Decimal Reduction Value

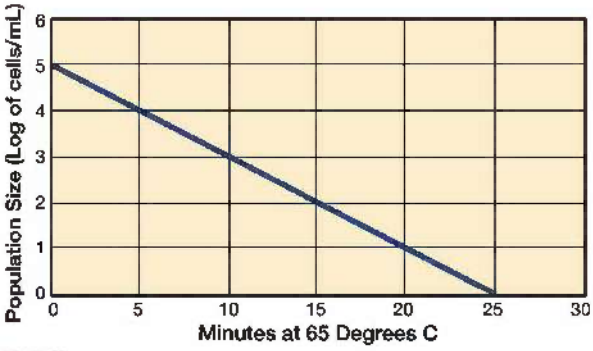
EXERCISE 6-6

Theory

The effects of excessive heat on bacteria range from disruption of cell membrane and protein function to complete combustion of the cell and all its components. Temperatures above 60°C are sufficient to kill most vegetative cells, whereas bacterial spores may survive temperatures greater than 100°C.

The time needed to kill a specific number of microorganisms at a specific temperature is called **thermal death time (TDT)**. Because microbial death occurs exponentially—that is, the number of *dead* cells increases exponentially—TDT can be expressed in logarithmic terms. Figure 6.16 illustrates a typical death curve where the log of population size (in cells per milliliter) is plotted against time at a specific temperature. This hypothetical organism, in a suspension of 10^5 cells/mL, survives 25 minutes at 65°C.

One variation of TDT, commonly used by the food industry, is the **decimal reduction value (D value)**. The D value is the time (in minutes at a specific temperature) needed to reduce the number of viable organisms in a given population by 90%—which is one logarithmic cycle. For example, one logarithmic cycle reduction of cell density in a population could be from 1.2×10^7 cells/mL to 1.2×10^6 cells/mL. For some people, it is easier to conceptualize when those numbers are converted to base 10 logarithmic terms: the population was reduced from $10^{7.08}$ to $10^{6.08}$ cells/mL. The temperature for the D value is customarily added as a subscript (e.g., D_{60}, D_{121}, etc.).



6.16 Thermal Death Time (TDT) Curve ◆ With an original cell density of 10^5 cells/mL, this hypothetical population survives 25 minutes at 65°C. Notice that it takes 5 minutes to reduce the population size by a factor of 10, *regardless of the magnitude of the population*. What would be the TDT if the population had started at a density of 10^6 cells/mL?

D value is useful because it mathematically represents death rate as a constant for a species under particular circumstances and is independent of population size. An organism with a D_{60} value of 4 takes 4 minutes at 60°C to reduce a population by 90%, regardless of the starting number of cells. Mathematically, D value is defined as:

$$D_T = \frac{t}{\log_{10} x - \log_{10} y}$$

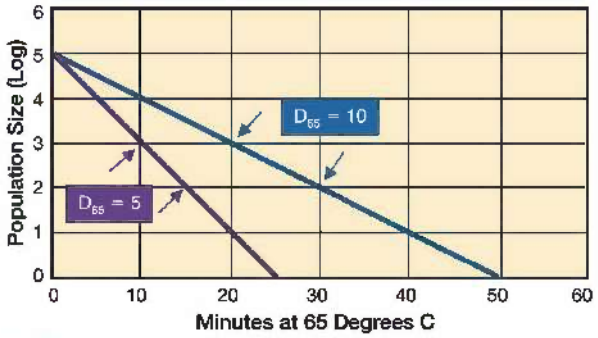
where

- T = temperature
- t = time in minutes
- x = cell density (cells per milliliter) before exposure to heat
- y = cell density after exposure to heat

For example, if a population of 1.7×10^5 cells heated at 60°C for 10 minutes was reduced to 2.6×10^3 cells, the D_{60} value would be 5.49 minutes.

$$D_{60} = \frac{10 \text{ min.}}{\log_{10} 1.7\times10^5 - \log_{10} 2.6\times10^3} = \frac{10 \text{ min.}}{5.23\times3.41} = 5.49 \text{ min.}$$

D value also can be plotted on a TDT standard curve, as shown in Figure 6.17. Either value (D value or TDT) can be determined from the other. A calculated D value requires two population density measurements—one before heating and one after heating. This produces two data points on the graph that can be used to construct a straight line TDT curve. Conversely, a TDT curve (determined by measuring the total time needed to kill the entire population) reveals the D value at the points (shown by arrows) where the curve intersects log values from the *y*-axis. The log values on the *y*-axis represent exponents for a base_{10} logarithmic scale. In other words, 1 is interpreted as 10^1, 2 is 10^2, 3 is 10^3 cells per milliliter, etc.



6.17 TDT Curves of Two Populations ♦ Species A (purple) takes 5 minutes to reduce its population by 90% (10^3 to 10^2 cells) whereas species B (blue) takes 10 minutes. The graph can be constructed either by calculating the D value and extrapolating from two data points (shown by the arrows) or by plotting the TDT curve and determining D value from the points of intersection with log values. Both methods produce a straight line. As you can see, D value is independent of population size.

Application

This exercise is used to determine microorganismal thermal death time (TDT) and decimal reduction value at 60°C.

6

In This Exercise

You will determine the D_{60} values of *Escherichia coli* and *Staphylococcus epidermidis* cultures using both methods described above. Your instructor will begin by adding bacteria to a broth maintained at 60°C. This is t_0—the beginning of the experiment. At this time and again at 10 minutes (t_{10}), a sample will be removed from the flask, diluted, and plated to perform viable counts to establish the density of the population.

In addition, beginning at t_1, samples will be removed from the flask at 1-minute intervals for 30 minutes (t_{30}). The samples will be used to inoculate nutrient broths. After a 48-hour incubation period, the nutrient broths will be examined for growth. The first broth in the sequence to produce no bacterial growth will establish the thermal death time of the population. The accumulated data then will be used to plot and calculate the D_{60} values of each organism.

In an attempt to divide the workload equitably and to allow time for all dilutions, transfers, and platings, the class will be divided into four groups, each of which will be further divided into a Plate Subgroup and a Broth Subgroup.

- Group One will be responsible for the t_0 platings of *E. coli,* and the inoculations of nutrient broths 1 through 15.
- Group 2 will be responsible for the t_{10} platings of *E. coli*, and the inoculations of nutrient broths 16 through 30.
- Group 3 will be responsible for the t_0 platings of *S. epidermidis,* and inoculations of nutrient broths 1 through 15.
- Group 4 will be responsible for the t_{10} platings of *S. epidermidis*, and the inoculations of nutrient broths 16 through 30.

✓ Materials

Per Class

- ☐ Stopwatch
- ☐ Thermometers
- ☐ Two flasks, each containing 49.0 mL nutrient broth and maintained at 60°C in a water bath
- ☐ 24-hour broth cultures of:
  - *Escherichia coli*
  - *Staphylococcus epidermidis*

Per Student Group

- ☐ Seven nutrient agar plates
- ☐ 16 nutrient broth tubes
- ☐ Sterile 0.1 mL, 1.0 mL, 5.0 mL, and 10.0 mL pipettes
- ☐ Sterile dilution tubes with caps (large enough to hold 10.0 mL)
- ☐ 75 mL of sterile deionized water in a small covered flask
- ☐ Screw-cap jar (with lid) of alcohol and bent glass rod for spreading organisms
- ☐ (Optional) bucket of crushed ice
- ☐ Pipette canister for pipette disposal

Procedure

Preliminary Notes

- This procedure requires knowledge of dilutions and calculations, explained fully in Exercise 6-1, Standard Plate Count. If you have not completed a standard plate count, or you have any doubt about your ability to complete this exercise, we suggest that you read Exercise 6-1 carefully and do some or all of the practice problems.
- This procedure uses the spread plate technique described in Exercise 1-5. Refer to it as needed.
- The Plate Subgroups will need at least two or three students for labeling tubes and plates, performing the serial dilution, and spreading plates.
- The Broth Subgroups will need one or two students to label and inoculate the 15 nutrient broths, keep track of time, and mix the broth prior to all transfers.
- The dilution/plating process can be slow for novices, so we recommend keeping dilution broths on ice until they can be plated to minimize bacterial reproduction.

- Page 855 in Appendix F provides an alternate procedure for using digital micropipettes.

Lab One: Group 1 (t_0)

Plate Subgroup

1. Obtain all plating materials. Place seven sterile test tubes in a rack. Label them 1 through 7. Add 9.0 mL of sterile water to tubes 2 through 7 and cover them until needed. You may want to keep them on ice throughout the upcoming dilutions.
2. Label seven nutrient agar plates 1 through 7.
3. When your instructor adds 1.0 mL of the *E. coli* culture to the heated flask, he/she will start the stopwatch. This is t_0.
   - Immediately swirl the flask to mix its contents. Remove 1.5 mL of broth from the flask and transfer it to tube 1. If possible, do this step without removing the flask from the water bath. Continue with the serial dilution below.
   - Transfer 1.0 mL from tube 1 to tube 2 and mix. Properly discard the pipette in the pipette canister. Use a fresh, sterile pipette for each transfer from here forward.
   - Transfer 1.0 mL from tube 2 to tube 3; mix.
   - Transfer 1.0 mL from tube 3 to tube 4; mix.
   - Transfer 1.0 mL from tube 4 to tube 5; mix.
   - Transfer 1.0 mL from tube 5 to tube 6; mix.
   - Transfer 1.0 mL from tube 6 to tube 7; mix.
   - Transfer 0.1 mL from tube 1 to plate 1 and spread according to the spread plate procedure.
   - Transfer 0.1 mL from tube 2 to plate 2 and spread.
   - Transfer 0.1 mL from tube 3 to plate 3 and spread.
   - Transfer 0.1 mL from tube 4 to plate 4 and spread.
   - Transfer 0.1 mL from tube 5 to plate 5 and spread.
   - Transfer 0.1 mL from tube 6 to plate 6 and spread.
   - Transfer 0.1 mL from tube 7 to plate 7 and spread.
4. Calculate the volume of original sample transferred to each plate (volume plated × dilution) and enter it in Table 1 on the data sheet, page 511. If you did Exercise 6-1, you have calculated original sample volumes before!
5. Tape the plates together, invert, and incubate them at 35 ± 2°C for 48 hours.

Broth Subgroup

1. Obtain all broth transfer materials. Label 15 nutrient broth tubes t_1 through t_{15}. Label the 16th tube "Control." Enter these numbers in the appropriate boxes in Table 2 on the data sheet.
2. When your instructor adds 1.0 mL of the *E. coli* culture to the heated flask, he/she will start the stopwatch. This is t_0. Slightly before 1 minute (t_1), gently swirl the flask to mix the broth. At exactly t_1, transfer 0.1 mL from the flask to the appropriately labeled nutrient broth tube. Properly discard the pipette in the pipette canister.
3. Repeat this procedure at t_2 through t_{15}. Be sure to mix the broth before each transfer, and use a fresh, sterile pipette.
4. Incubate all broths at 35 ± 2°C for 48 hours.

Lab One: Group 2 (t_{10})

Plate Subgroup

1. Obtain all plating materials. Place six sterile test tubes in a rack and label them 1 through 6. Add 9.0 mL of sterile water to tubes 2 through 6 and cover them until needed. You may want to keep them on ice throughout the upcoming dilutions.
2. Label seven nutrient agar plates 1 through 7, respectively.
3. When your instructor adds 1.0 mL of the *E. coli* culture to the heated flask, he/she will start the stopwatch. This is t_0 and your work will not begin until 10 minutes later (t_{10}).
   - At t_{10}, swirl the flask to mix its contents. After the broth group withdraws their t_{10} sample, remove 2.5 mL of broth and transfer it to tube 1. If possible, do this step without removing the flask from the water bath. Continue with the serial dilution below.
   - Transfer 1.0 mL from tube 1 to tube 2 and mix. Discard the pipette. Use a fresh, sterile pipette for each transfer from here forward.
   - Transfer 1.0 mL from tube 2 to tube 3; mix.
   - Transfer 1.0 mL from tube 3 to tube 4; mix.
   - Transfer 1.0 mL from tube 4 to tube 5; mix.
   - Transfer 1.0 mL from tube 5 to tube 6; mix.
   - Transfer 1.0 mL from tube 1 to plate 1 and spread according to the spread plate procedure.
   - Transfer 0.1 mL from tube 1 to plate 2 and spread.
   - Transfer 0.1 mL from tube 2 to plate 3 and spread.
   - Transfer 0.1 mL from tube 3 to plate 4 and spread.
   - Transfer 0.1 mL from tube 4 to plate 5 and spread.
   - Transfer 0.1 mL from tube 5 to plate 6 and spread.
   - Transfer 0.1 mL from tube 6 to plate 7 and spread.

4. Calculate the volume of original sample transferred to each plate (volume plated × dilution) and enter it in Table 1 on the data sheet, page 511. If you did Exercise 6-1, you have calculated original sample volumes before!
5. Tape the plates together, invert, and incubate them at 35 ± 2°C for 48 hours.

Broth Subgroup

1. Obtain all broth transfer materials. Label 15 nutrient broth tubes t_{16} through t_{30}. Label the 16th tube "Control." Enter these numbers in the appropriate boxes in Table 2 on the data sheet.
2. When your instructor adds 1.0 mL of the *E. coli* culture to the heated flask, he/she will start the stopwatch. This is t_0 and your work will not begin until about 15 minutes later. Slightly before t_{16} (16 minutes after the start), gently swirl the broth flask and at exactly t_{16}, transfer 0.1 mL from the flask to the appropriately labeled nutrient broth tube. Discard the pipette in the pipette canister.
3. Repeat this procedure at t_{17} through t_{30}. Be sure to mix the broth before each transfer, and use a fresh, sterile pipette.
4. Incubate all broths at 35 ± 2°C for 48 hours.

Lab One: Group 3 (t_0)

Plate Subgroup

Using the *S. epidermidis* culture, follow the Group 1 Plate Subgroup instructions.

Broth Subgroup

Using the *S. epidermidis* culture, follow the Group 1 Broth Subgroup instructions.

Lab One: Group 4 (t_{10})

Plate Subgroup

Using the *S. epidermidis* culture, follow the Group 2 Plate Subgroup instructions.

Broth Subgroup

Using the *S. epidermidis* culture, follow the Group 2 Broth Subgroup instructions.

Lab Two: All Groups

1. Remove all broths and plates from the incubator.
2. Pick the plate containing between 30 and 300 colonies. It is the countable plate and there should be only one. All others should be either TFTC (too few to count) or TNTC (too numerous to count). Label them as such, and enter this information in Table 1 on the data sheet.
3. If you have not already done so, count the colonies on the countable plate and enter the number in Table 1 on the data sheet.
4. Calculate the population density of your group's organism for your specified time (t_0 or t_{10}), using the following formula:

$$\text{Cell density (CFU/mL)} = \frac{\text{CFU}}{\text{Original sample volume}}$$

(*Note*: "Original" in this formula refers to the *sample being heated,* not the culture tube used to inoculate it. As described in Exercise 6-1, calculate the original sample volume being transferred to a plate by multiplying the *dilution* of the sample by its *volume*.)

5. Enter your results in Table 1 and again in Table 3 on the data sheet, pages 511 and 512. Also, be sure to convert your results to logarithmic form, and enter that in Table 3 as well.
6. Examine the 15 broths for growth, comparing each one to the uninoculated control (tube #16). Any turbidity is read as positive for growth. Enter your results in Table 2 on the data sheet. Circle the earliest time that no turbidity appears (i.e., growth did not occur) and enter this time in Table 3.
7. Collect the data from other groups and enter these in Table 3 on the data sheet. Your instructor will likely provide a means of efficiently distributing this information.
8. Follow the directions on the data sheet to plot *and* calculate the D values of both organisms.
9. Answer the questions on the data sheet.

References

Ray, Bibek. Chap. 31 in *Fundamental Food Microbiology*, 2nd ed. Boca Raton, FL: CRC Press, 2001.

National Canners Association Research Laboratories. *Laboratory Manual for Food Canners and Processors*, Vol. 1: Microbiology and Processing. Westport, CT: AVI Publishing Co., 1968.

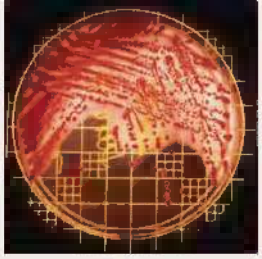
Stumbo, Charles Raymond. Chap. 7 in *Thermobacteriology in Food Processing*, 2nd ed. New York: Academic Press, 1973.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-6

Thermal Death Time Versus Decimal Reduction Value

DATA AND CALCULATIONS

It works best to reproduce the data sheet tables on the blackboard or a transparency so that the entire class can enter and share data.

1 On Day 1, fill in the name of the organism you are testing and the first two rows of Table 1. On Day 2, fill in all the remaining boxes. For plates containing fewer than 30 or more than 300 colonies, enter TFTC or TNTC, respectively. Enter your calculated cell density in the appropriate part of Table 3.

Table 1—Group Plate Data

Organism:							
Plate Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Plate Number							
Volume of Original Sample Plated (vol. plated × dilution)							
Colonies Counted (CFU)							
Cell Density (CFU/mL)							

2 On Day 1, fill in the organism you are testing and the time subscripts according to your part of the experiment. That is, either t_1 through t_{15} or t_{16} through t_{30}. On Day 2, enter "G" for growth and "NG" for no growth in the broth tubes. Circle the first time at which no growth appears in the broth and enter that time in Table 2.

Table 2—Group Broth Data

Organism:					
Time	**G/NG**	**Time**	**G/NG**	**Time**	**G/NG**
T_____		T_____		T_____	
T_____		T_____		T_____	
T_____		T_____		T_____	
T_____		T_____		T_____	
T_____		T_____		T_____	

3 On Day 2, enter class data in Table 3.

Table 3—Class Data

Organism	t_0		t_{10}		First Broth Without Growth (t_x)
	Cell Density (Cells/mL)	Log G_{10} of Cell Density	Cell Density (Cells/mL)	Log G_{10} of Cell Density	
E. coli					
S. epidermidis					

Perform the following for *each* organism.

Constructing the TDT curve

1 On the graph paper provided on page 515, construct a graph of Population size (log) versus Time at 60°C, as shown in Figure 6.17. To do this:

a. Enter a data point on the *y*-axis representing the log of the original cell density of the broth culture at t_0 (from Table 3).

b. Enter a second data point on the *x*-axis at the time when growth in the nutrient broth tube stopped—thermal death time (from Table 2).

c. Draw a straight line between the two points. This is the TDT curve for this culture at 60°C.

6

2 Use this curve for plotting the D value.

Plotting D value from TDT curve

3 Locate two successive log values on the *y*-axis below the highest population point for the organism, representing one complete log cycle (*e.g.*, 10^6 and 10^5, 10^5 and 10^4, etc.).

4 Draw a horizontal line from each of these points on the *y*-axis to where they intersect the TDT curve.

5 Draw a vertical line from these intersection points downward to where they intersect with the *x*-axis.

6 The horizontal distance between these two points reveals the time required to kill 90% of the population. This is the D value of the organism. Enter it in Table 4 in the box labeled "Plotted D_{60} value."

Table 4—Thermal Death Time Versus Decimal Reduction Value

Organism	Thermal Death Time (min.)	Plotted D_{60} Value (min.)	Calculated D_{60} Value (min.)
E. coli			
S. epidermidis			

Calculating D value

1 From Table 3, obtain the logs of the microbial cell densities before and after heating.

2 Use the following formula to calculate the D_{60} value of each organism.

$$D_T = \frac{t}{\log_{10} x - \log_{10} y}$$

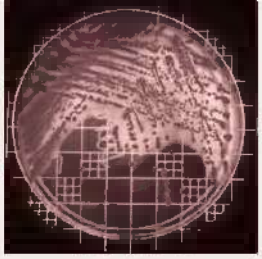
3 Enter your results in Table 4 in the box labeled "Calculated D_{60} Value."

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 6-6

(continued)

QUESTIONS

1 *Is the D value of either organism affected by the size of the population? Explain.*

2 *How much did your plotted and calculated values differ? How do you explain any differences?*

3 *Using the information from this exercise:*

a. *How long would it take to kill a population of* E. coli *with a density of* 10^3 *cells/mL?*

b. *How long to kill a population of* S. epidermidis *with the same density?*

4 *Because this is a quantitative procedure, why is it not necessary to know the volume of broth in the 30 nutrient broth tubes?*

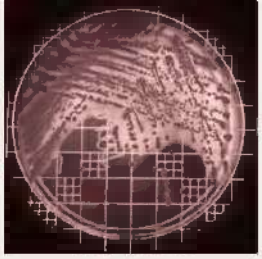
6

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

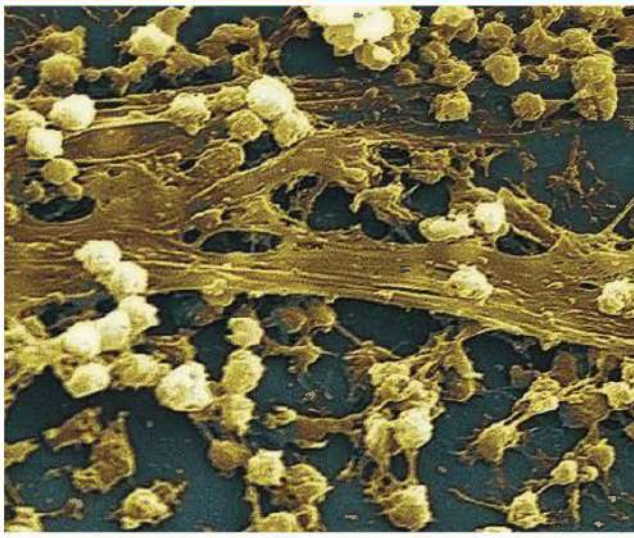


DATA SHEET 6-6

(continued)

SECTION 7

Medical Microbiology



The study and application of microbiological principles and those of medicine are inseparable. So, although this is not a medical microbiology manual, it nonetheless is devoted largely to the study of microorganisms and their relationship to human health. Our superficial treatment of medical microbiology in this section is not meant to represent a balanced body of information but, rather, is composed of exercises to augment those you have already done related to medical microbiology.

In this section you will perform a test to detect susceptibility to dental decay, do a quantitative test for an enzyme (lysozyme) that degrades Gram-positive cell walls, check the effectiveness of various antibiotics on sample bacteria, and demonstrate the formation of a medically important biofilm. These exercises are followed by two epidemiological exercises. In the first, you will use a standard resource—*Morbidity and Mortality Weekly Report* available from the Centers for Disease Control and Prevention (CDC) website—to follow the incidence of a disease of your choice over the course of two complete years. In the second exercise, you will simulate an epidemic outbreak in your class, determine the source of the outbreak, and perform epidemiological analyses of your class population.

The final three exercises in this section are a culmination of all you have done up to this point. You will be using skills developed in earlier exercises and apply them as a practicing microbiologist would. That is, you will be identifying unknown microbes from samples supplied to you by your instructor.

The opportunity to apply what you have learned to a real problem with practical applications is both challenging and exciting for most students. A word of caution, though. Your instructor will know what your unknown is and can tell you if your identification is correct. However, out of the classroom and in a professional laboratory no one can tell the microbiologist if he or she is correct or not. In fact, "identification" is more about finding out what the unknown *isn't,* rather than finding out with *certainty* what it *is*. There is an unspoken understanding that the unknown's identity is based on the best match between its test results and the accepted results for the same tests of the named species. The identification is provisional because other tests not run might lead to a different conclusion. Or, the unknown is a strain with genetic variability that makes it different from the majority of strains in that species. Finally, don't forget about those nasty false positive and false negative test results. Now, with that in mind…

The three exercises employ traditional methods of microbial identification. That is, you will use staining properties, cell morphology, cell arrangement, and biochemical test results to identify an unknown organism assigned to you. In each exercise, a flowchart will direct you in your choice of tests. Matching your unknown's results to the flowchart will lead you, by the process of elimination, to a provisional identification. This methodology will be applied to common species of Gram-negative rods (Exercise 7-7), Gram-positive cocci (Exercise 7-8), and Gram-positive rods (Exercise 7-9).

You have already done most tests in the flowcharts. Some, such as fermentation of sugars, use the same PR medium as Exercise 5-2, just with a different sugar. These are read as in Exercise 5-2. The same applies to decarboxylase media (Exercise 5-11).

EXERCISE
7-1

Snyder Test

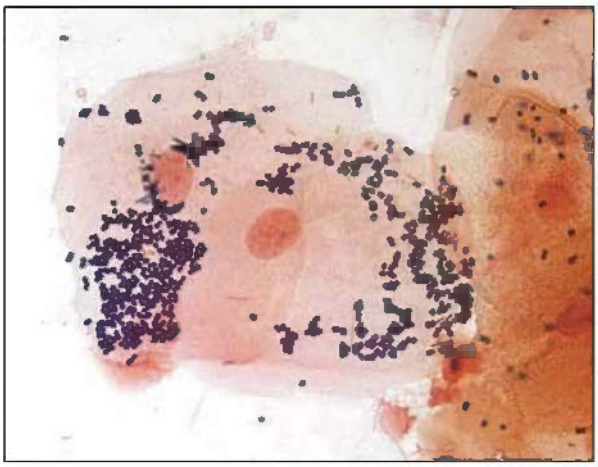
Theory

The oral cavity presents a variety of habitats for microbial growth, including the tongue, floor of the mouth, and buccal (cheek) and palatal surfaces. These surfaces are covered with stratified squamous epithelium that sheds on a regular basis, thus making heavy microbial colonization difficult. Then there are the teeth, the only organs in the body that present hard surfaces to the outside world. Microbial communities attach firmly to the teeth in **biofilms**, more commonly known as **dental plaque**. This firm attachment to teeth makes colonization more permanent and more abundant than on the oral epithelial surfaces.

Tooth biofilms are inhabited by a diverse assemblage of bacteria that become attached to a proteinaceous film covering teeth called a **pellicle**. Initial attachment of bacteria to the tooth surface involves many factors, but specific bacterial **adhesins** bind specifically to host protein receptors, which accounts in large part for the presence of microorganisms characteristic of dental plaque. Many of these microbes are fermenters of sugars, such as glucose and sucrose, and produce acid as an end product. It is the acid that demineralizes tooth enamel and results in **caries** (cavities). The worst offenders are *Streptococcus mutans*, other streptococci, *Actinomyces* species, and *Lactobacillus* species. Not surprisingly, the people who are most at risk are those with a high dietary intake of sugars and poor dental hygiene. To make matters worse, these organisms have evolved mechanisms to tolerate low pH conditions (pH of ~5.5) that inhibit growth of other less harmful bacteria.

Snyder test medium is formulated to favor the growth of oral bacteria (Fig. 7.1) and discourage the growth of other bacteria. This is accomplished by lowering the pH of the medium to 4.8. Glucose is added as a fermentable carbohydrate, and bromcresol green is the pH indicator. Lactobacilli and oral streptococci survive these harsh conditions, ferment the glucose, and lower the pH even further. The pH indicator, which is green at or above pH 4.8 and yellow below, turns yellow in the process. Development of yellow color in this medium, therefore, is evidence of fermentation and, further, is highly suggestive of the presence of dental decay-causing bacteria (Fig. 7.2).

The medium is autoclaved for sterilization, cooled to just over 45°C, and maintained in a warm-water bath until needed. The molten agar then is inoculated with a small amount of saliva, mixed well, and incubated for up to 72 hours. The agar tubes are checked at 24-hour intervals for any change in color. High susceptibility to dental caries is indicated if the medium turns yellow within 24 hours. Moderate and slight susceptibility are indicated by a change within 48 and 72 hours, respectively. No change by 72 hours is considered a negative result. These results are summarized in Table 7-1.



7.1 Gram Stain of Bacteria Harvested from Below the Gumline ♦ Note the predominance of Gram-positive cocci. Also present are a few Gram-negative rods and very few Gram-positive rods (*Lactobacillus*). Note also the size difference between the large epithelial cells in the center and the bacteria.

7.2 Snyder Test Results ♦ A positive result is on the left and a negative result is on the right.

TABLE 7-1 Snyder Test Results and Interpretations

Table of Results	
Result	**Interpretation**
Yellow at 24 hours	High susceptibility to dental caries
Yellow at 48 hours	Moderate susceptibility to dental caries
Yellow at 72 hours	Slight susceptibility to dental caries
Yellow at >72 hours	Negative

Application

The Snyder test is designed to measure susceptibility to dental caries (tooth decay), caused primarily by lactobacilli and oral streptococci.

In This Exercise

You will check your own oral microflora for its potential to produce dental caries.

Materials

Per Class

- ☐ Hot-water bath set at 45°C
- ☐ Broth cultures of *Lactobacillus delbrueckii*

Per Student

- ☐ 10 mL sterile beaker
- ☐ Sterile 1 mL pipettes with mechanical pipettor
- ☐ Canister for pipette disposal
- ☐ Three Snyder agar tubes

Medium Recipe

Snyder Test Medium

• Pancreatic digest of casein	13.5 g
• Yeast extract	6.5 g
• Dextrose	20.0 g
• Sodium chloride	5.0 g
• Agar	16.0 g
• Bromcresol green	0.02 g
• Distilled or deionized water	1.0 L
pH 4.6–5.0 at 25°C	

Procedure

Lab One

1. Collect a small sample of saliva (about 0.5 mL) in the sterile beaker.
2. Aseptically add 0.2 mL of the sample to a molten Snyder agar tube (from the water bath), and roll it between your hands until the saliva is distributed uniformly throughout the agar. Label the culture with your name and date of inoculation.
3. Inoculate one Snyder test medium with a few drops of *L. delbrueckii* and roll it between your hands as in step 2. Label the culture with the organism's name and date of inoculation.
4. Allow the agar to cool to room temperature in both tubes. Do not slant.
5. Incubate with an uninoculated control at 35 ± 2°C.
6. Examine the tubes for color changes at 24-hour intervals for up to 72 hours.

Lab Two (Or whenever the tube shows a positive result)

1. Record your results in the table provided on the data sheet, page 521.

References

Quivey, Robert G. Chap. 11, Caries in *Oral Microbiology and Immunology.* Richard J. Lamont, Robert A. Burne, Marilyn S. Lantz, and Donald J. LeBlanc, eds. Washington, DC: ASM Press, 2006.

Scannapieco, Frank A. Chap. 3, The Oral Environment in *Oral Microbiology and Immunology.* Richard J. Lamont, Robert A. Burne, Marilyn S. Lantz, and Donald J. LeBlanc, eds. Washington, DC: ASM Press, 2006.

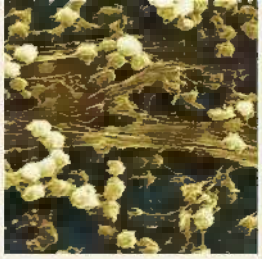
Zimbro, Mary Jo and David A. Power, eds. Page 517 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media.* Sparks, MD: Becton Dickinson and Co., 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-1

Snyder Test

OBSERVATIONS AND INTERPRETATIONS

1 Record the color of the *Lactobacillus delbrueckii* culture at each time interval in the table below.

2 Record the color of your saliva culture at each time interval in the table below. Refer to Table 7-1, page 520, when recording and interpreting your results below.

3 Record the color of the uninoculated control at each time interval in the table below.

	Time		
	24 Hrs.	48 Hrs.	72 Hrs.
L. delbrueckii culture			
Saliva sample			
Uninoculated control			

Based on the results of this test, I have a _____ susceptibility to dental caries.

QUESTIONS

1 *As demonstrated by this exercise,* Lactobacillus *species ferment glucose (dextrose). Considering their role in fermentation of milk, what other ingredient in Snyder test medium would you expect* Lactobacillus *species to digest?*

2 *Using Snyder test medium as a guide, what kinds of dietary items would likely increase the number of lactobacilli and streptococci in saliva (and in turn, on tooth biofilms)?*

3 *Why isn't the molten Snyder test agar allowed to solidify as a slant?*

4 *What purpose does the uninoculated control serve?*

5 *What purpose does the* L. delbrueckii *culture serve?*

7

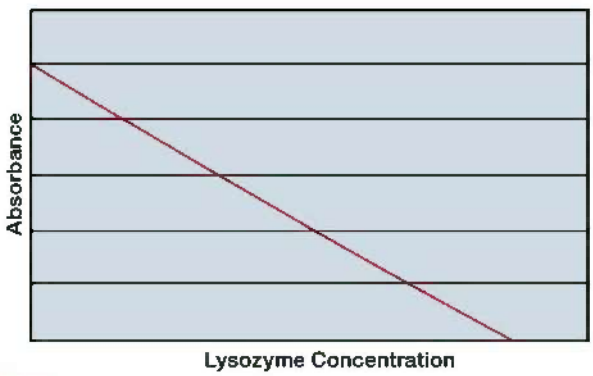
EXERCISE
7-2
Lysozyme Assay

Theory

Lysozyme was discovered in 1922 by Alexander Fleming (who also discovered penicillin) as a component of nasal mucus harvested from a patient with a cold that inhibited bacterial growth. It is an enzyme that occurs naturally in egg albumin and normal body secretions such as tears, saliva, mucus, and urine. It is also present inside vesicles within phagocytic polymorphonuclear granulocytes (PMNs). The enzyme provides limited protection from bacterial infection by breaking bonds in the cell wall's **peptidoglycan.** Peptidoglycan is composed of long glycan chains of the alternating subunits *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), cross-linked by peptides. Lysozyme functions by breaking the β-1,4 glycosidic linkages between the NAG and NAM subunits. It is more effective on Gram-positive organisms than on Gram-negatives, because it can't cross the latter's outer membrane.

A lysozyme assay measures the ability of a sample to lyse cells of the substrate organism *Micrococcus lysodeikticus*. Cell lysis occurs as a result of damage caused by the lysozyme and the hypotonic diluent used to dilute the samples.

In this exercise you will measure the lysozyme concentrations in a variety of body fluids. To do this you will first construct a standard curve (see Fig. I.11, page 11, and Fig. 7.3) for comparison. First, you will mix diluted lysozyme samples of known concentration with an equal part of the bacterial substrate solution, then take an absorbance reading (with a spectrophotometer) of each after 20 minutes. You will then plot absorbance vs. lysozyme concentration to produce the standard curve. Dilutions of natural fluid samples can then be prepared and mixed with the same amount of bacterial substrate solution as used in development of the standard curve. Read the absorbance after 20 minutes. The light absorbance values of the samples can then be used to interpolate the lysozyme concentrations from the standard curve.



7.3 Lysozyme Standard Curve ◆ This is a typical standard curve illustrating an inverse correlation between light absorbance and lysozyme concentration (also see Fig. I.11, page 11). As the concentration of lysozyme increases and lysis of substrate cells increases, the solution in the tube absorbs less light. Lysozyme concentration in a sample can be estimated by finding the lysozyme concentration that corresponds to the absorbance value of the sample.

Application

This exercise is used to estimate the relative concentrations of lysozyme in body fluids.

In This Exercise

The work for this exercise will be divided among several groups. One group will perform the dilutions of known lysozyme concentrations for construction of the standard curve. Other groups will dilute and test samples of tears, saliva, urine, and/or other body fluids as determined by your instructor. The data then will be shared among lab groups to complete the data sheet, page 525.

✓ Materials[1]

Per Student Group

- ☐ Spectrophotometer with cuvettes
- ☐ Timer
- ☐ Digital pipettes (100–1,000 µL) with sterile tips
- ☐ Receptacle for pipette tip disposal
- ☐ 1 mL and 5 mL pipettes
- ☐ Propipettes
- ☐ Parafilm
- ☐ Lysozyme buffer (20 mL for Group 1, 10 mL for other groups)
- ☐ Lysozyme substrate—*Micrococcus lysodeikticus* (ATCC No. 4698)—in solution measuring 10.0% transmittance (15 mL for Group 1, 5 mL for other groups)
- ☐ Lysozyme dilutions shown in Table 7-2 (2.5 mL of each for Group 1)

[1] **Note to instructor:** One liter of buffer is sufficient to make the lysozyme dilutions and substrate solution for a class of 30 to 35 students.

TABLE 7-2 Lysozyme Dilutions

Table of Results		
Tube	Contains	In
A	0.15625 (0.156) mg Lysozyme	100 mL Buffer
B	0.3125 (0.313) mg Lysozyme	100 mL Buffer
C	0.625 mg Lysozyme	100 mL Buffer
D	1.25 mg Lysozyme	100 mL Buffer
E	2.5 mg Lysozyme	100 mL Buffer
F	5.0 mg Lysozyme	100 mL Buffer

Procedure

Note: This exercise is written so that either glass pipettes or digital pipettors can be used.

Group 1: Standard Curve

1. Turn on the spectrophotometer and allow it to warm up for a few minutes. Set the wavelength to 540 nm and, if it is digital, set it to absorbance. Be sure the filter is set properly for the wavelength used. (For directions on spectrophotometer use, see Appendix E.)
2. Obtain seven cuvettes. Transfer 2.0 mL (2,000 µL) of lysozyme substrate (*M. lysodeikticus*) solution to each of six cuvettes; transfer 4.0 mL (4,000 µL) straight lysozyme buffer to the seventh.
3. Blank the spectrophotometer with the cuvette containing the straight buffer. Continue to check the setting throughout the procedure and blank the machine as needed.
4. Transfer 2.0 mL (2,000 µL) of the 0.15625 mg/100 mL lysozyme dilution[2] to a substrate-containing cuvette. Mark the time in the Standard Curve table on the data sheet. This is t_0 for this concentration. (*Note*: Time is critical for this part of the exercise. The absorbance of each lysozyme/substrate mixture must be read at ***exactly 20 minutes***. Therefore, begin timing each transfer at the moment it is done.)
5. Continue transferring 2.0 mL (2,000 µL) of each lysozyme dilution to different substrate-containing cuvettes. Mark each time on the data sheet.
6. Take the absorbance readings at t_{20} for each mixture. Record your results in the table on the data sheet.
7. Share these data with the class.

[2] Lysozyme concentrations are shown to more significant figures than are practical to measure only to illustrate that each is one-half of the next most concentrated. When preparing the solutions, masses can be measured to three decimal places (or less, depending on the precision of your balance).

Other Groups: Determination of Lysozyme in Body Fluid Samples

1. Turn on the spectrophotometer and allow it to warm up for a few minutes. Set the wavelength to 540 nm and, if it is digital, set it to absorbance. Be sure the filter is set properly for the wavelength used. (For directions on spectrophotometer use, see Appendix E.)
2. Obtain four cuvettes and all other necessary material for your body fluid sample.
3. Collect your assigned body fluid according to your teacher's instructions.
4. Add 0.2 mL (200 µL) undiluted sample to a cuvette. Add 3.8 mL (3,800 µL) lysozyme buffer. Mix well. Use this mixture to blank the spectrophotometer. Check this setting frequently throughout the exercise, and adjust as necessary.
5. Add 0.2 mL (200 µL) undiluted sample to a cuvette. Add 1.8 mL (1,800 µL) lysozyme buffer. Mix. This is a 10^{-1} dilution.
6. Transfer 0.2 mL (200 µL) from the 10^{-1} dilution to the third cuvette. Add 1.8 mL (1,800 µL) lysozyme buffer. Mix. This is a 10^{-2} dilution.
7. Add 1.8 mL (1,800 µL) lysozyme substrate to the 10^{-1} cuvette and mix well. Mark the time on the data sheet. This is t_0 for this mixture. (*Note*: Time is critical for this part of the exercise. The absorbance of each body fluid/substrate mixture must be read at ***exactly 20 minutes***. Therefore, begin timing each transfer at the moment it is done.)
8. Add 2.0 mL (2,000 µL) lysozyme substrate to the 10^{-2} cuvette. Mix well. Mark the time on the data sheet. This is t_0 for this mixture.
9. Take the absorbance readings at t_{20} for each mixture. Record your results in the table on the data sheet.
10. Share these t_{20} absorbance values of body fluids with the class.

References

DIFCO Laboratories. Page 515 in *DIFCO Manual*, 10th ed. Detroit: DIFCO Laboratories, 1984.

Fleming, A. "Series B, Containing Papers of a Biological Character," Proceedings of the Royal Society of London 93, no. 653 (May 1, 1922): 306–317.

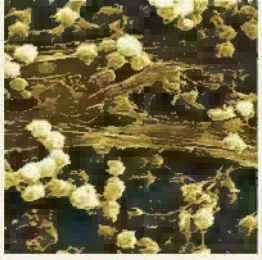
Sprott, G. Dennis, Susan F. Koval, and Carl A. Schnaitman. Page 78 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-2

Lysozyme Assay

OBSERVATIONS AND INTERPRETATIONS

1 Enter the standard curve data from Group 1.

Lysozyme Concentration	Actual Time of Transfer (t_0)	Light Absorbance at t_{20}
0.15625 mg/100 mL		
0.3125 mg/100 mL		
0.625 mg/100 mL		
1.25 mg/100 mL		
2.5 mg/100 mL		
5.0 mg/100 mL		

2 Enter class data for all samples.

Body Fluid	Actual Time of Transfer (t_0)		Light Absorbance at t_{20}	
	10^{-1}	10^{-2}	10^{-1}	10^{-2}

3 Using the information in the tables and the graph paper provided, construct a standard curve of lysozyme concentration (x-axis) versus absorbance (y-axis). Don't forget to label the axes and title the graph.

4 Using the standard curve, estimate the concentration of lysozyme in each diluted sample.

5 Calculate the original concentration (before dilution) of lysozyme in all samples, using the following formula (where OC is original concentration, LC is the lysozyme concentration obtained from the standard curve, and D is the dilution):

$$OC = \frac{LC}{D}$$

QUESTIONS

1 *Lysozyme alone is not enough to kill bacterial cells. What other condition must be met in order for cell death to occur?*

2 *Why is lysozyme less effective against Gram-negative bacteria than Gram-positives?*

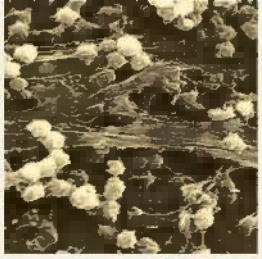
3 *Assuming the conditions are present for lysozyme to be effective, would it work on actively growing cells? Nongrowing cells?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-2

(continued)

Antimicrobial Susceptibility Test: Disk Diffusion (Kirby-Bauer) Method

EXERCISE 7-3

Theory

Antibiotics are natural antimicrobial agents produced by microorganisms. One type of penicillin, for example, is produced by the mold *Penicillium notatum*. Today, because many agents that are used to treat bacterial infections are synthetic, the terms **antimicrobials** or **antimicrobics** are used to describe all substances used for this purpose.

In 1966 Alfred Bauer, William Kirby, and associates first published a standardized disk diffusion method of testing the effectiveness of over 20 antimicrobial chemicals. Their methodology, which bears their names, has become a valuable tool for establishing the effectiveness of antimicrobics against pathogenic microorganisms in clinical laboratories.

In the test, antimicrobic-impregnated paper disks are placed on an agar plate inoculated to form a bacterial lawn. The plates are incubated to allow growth of the bacteria and time for the agent to diffuse into the agar. As the drug moves through the agar, it establishes a concentration gradient. If the organism is susceptible to it, a clear zone—**zone of inhibition**—will appear around the disk where the concentration is high enough to stop growth (Fig. 7.4). The junction of the zone of inhibition with growth is critical to interpretation of the test. It is at this junction where the concentration of antimicrobic has become too low to effectively stop growth. This junction represents the **minimum inhibitory concentration (MIC)** for that particular strain.

The basic disk diffusion test does not quantify the MIC (though variations are available that do this), but rather utilizes the zone of inhibition's size, which depends upon the sensitivity of the organism to the specific antimicrobial agent (among other factors). Interestingly, the clear zone does not necessarily mean that the bacteria have been killed. Drugs that kill the organism are said to be **bactericidal**, but other drugs are **bacteriostatic**; that is, they stop the bacteria from dividing, but do not kill them.

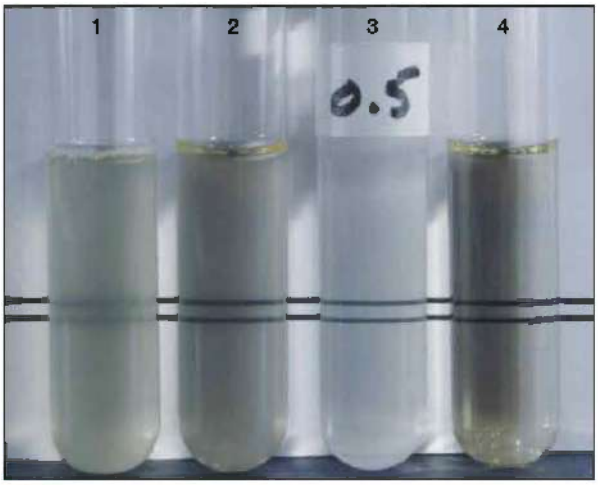
All aspects of the Kirby-Bauer procedure are standardized to ensure reliable results. Therefore, care must be taken to adhere to these standards. Mueller-Hinton agar, which has a pH between 7.2 and 7.4, is poured to a depth of 4 mm in either 150 mm or 100 mm Petri dishes. The depth is important because of its effect upon the diffusion. Thick agar slows lateral diffusion and thus produces smaller zones than plates held to the 4 mm standard. Inoculation is made with a broth culture, generally incubated 4 to 6 hours to bring it into exponential phase, and subsequently diluted to match a **0.5 McFarland turbidity standard**, which corresponds to a cell density between 1 and 2 $\times$ 10^8 CFU/mL (Fig. 7.5). Alternatively, a spectrophotometer can be used to bring the broth culture to an absorbance of 0.08 to 0.10 at 625 nm in a 1 cm cuvette, which corresponds to the 0.5 McFarland standard.

The U.S. Food and Drug Administration establishes the disk concentration of each agent, which is printed on the disk along with a code indicating the chemical agent. Disks are then dispensed onto the inoculated plate (Fig. 7.6), which are incubated at 35 $\pm$ 2°C for 16 to 18 hours. After incubation, the plates are removed and the zone diameters are measured in millimeters (Fig. 7.7) and compared to a standardized table of results (Table 7.3).

The Clinical Laboratory Standards Institute (CLSI) in Wayne, Pennsylvania, has performed extensive research (and continues to do so) to establish the zone diameter

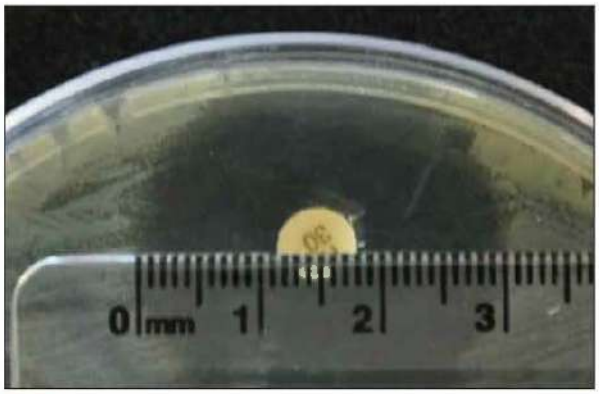
7

7.4 Disk Diffusion Test of Methicillin-Resistant *Staphylococcus aureus* (MRSA) ◆ This plate illustrates the effect of (clockwise from top outer right) Nitrofurantoin (F/M300), Norfloxacin (NOR 10), Oxacillin (OX1), Sulfisoxazole (G 0.25), Ticarcillin (TIC 75), Trimethoprim-Sulfamethoxazole (SXT), Tetracycline (TE 30), Ceftizoxime (ZOX 30), Ciprofloxacin (CIP 5), and (inner circle from right) Penicillin (P 10), Vancomycin (VA 30), and Trimethoprim (TMP 5) on Methicillin-resistant *Staphylococcus aureus* isolated from a patient.



7.5 McFarland Standards ♦ This is a comparison of a McFarland turbidity standard to three broths having varying degrees of turbidity. Each of the 11 McFarland standards (0.5 to 10) contains a specific percentage of precipitated barium sulfate to produce turbidity. In the Kirby-Bauer procedure, the test culture is diluted to match the 0.5 McFarland standard (roughly equivalent to 1.5×10^8 CFU/mL) before inoculating the plate. Comparison is made visually by placing a card with sharp black lines behind the tubes. Tube 3 is the 0.5 McFarland standard. Notice that the turbidity of Tube 2 matches the McFarland standard exactly, whereas Tubes 1 and 4 are too turbid and too clear, respectively. Alternatively, cultures may be standardized with a spectrophotometer. They should have an absorbance of 0.08 to 0.10 at 625 nm in a 1 cm cuvette.

7.6 Disk Dispenser ♦ This antibiotic disk dispenser is used to deposit disks uniformly on a Mueller-Hinton agar plate.



7.7 Measuring the Antimicrobial Susceptibility Zones ♦ Using a metric ruler and a dark, nonreflective background, measure the diameter of each clearing. Standards for comparison are given in millimeters (mm). This zone is 29 mm in diameter.

interpretive standards in Table 7-4—and there are many more antimicrobial agent/pathogen combinations we have not reproduced! These are published in a document titled, "M02–A11: Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard–Eleventh Edition (2012)" and updated in "M100–S24: Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Fourth Informational Supplement (2014)." Simply put, zone diameter breakpoints for resistance and susceptibility to a particular antimicrobial agent are determined after running a large number of specimens in which both the MIC and the zone diameter is established. The zone diameter below which all resistant strains fall is the **resistance breakpoint**. Likewise, the zone diameter above which all susceptible strains fall is the **susceptibility breakpoint**. In some cases, there may be an intermediate range of zone diameters in which both resistant and susceptible strains fall.

Application

The disk diffusion test is a standardized method used to measure the effectiveness of antibiotics and other chemotherapeutic agents on pathogenic microorganisms. In many cases, it is an essential tool in prescribing appropriate treatment.

In This Exercise

You will test the susceptibility of *Escherichia coli* and *Staphylococcus aureus* strains to penicillin, chloramphenicol, tetracycline (optional), trimethoprim, streptomycin (optional), and ciprofloxacin. These antibiotics were chosen because they exhibit different modes of action on bacterial cells (Table 7-3).

TABLE 7-3 Antibiotic Targets and Resistance Mechanisms ♦ Not all antibiotics affect cells in the same way. Some attack the bacterial cell wall, and others interfere with biosynthesis reactions. Resistance mechanisms can be broken down into four main categories: (a) altered target such that the antibiotic no longer can interact with the cellular process, (b) an alteration in how the drug is taken into the cell, (c) enzymatic destruction of the drug, and (d) development or increased activity of an efflux mechanism.

Antibiotic	Cellular Target	Resistance Mechanism
Chloramphenicol	Prevents peptide bond formation during translation	1. Poor uptake of drug 2. Inactivation of drug
Ciprofloxacin	Interferes with DNA replication	1. Altered target 2. Poor uptake of drug
Penicillin	Inhibits cross-linking of the cell wall's peptidoglycan	One or more of: 1. Altered target 2. Poor uptake of drug 3. Production of β-lactamases
Streptomycin	Blocks initiation complex formation in protein synthesis	1. Altered target
Tetracycline	Blocks attachment of aminoacyl tRNA to A site on ribosome	1. Efflux mechanism
Trimethoprim	Inhibits purine and pyrimidine synthesis	1. Altered target

TABLE 7-4 Zone Diameter Interpretive Chart

Antibiotic	Organism(s)	Code	Disk Potency	Zone Diameter Interpretive Standards (mm)		
				Susceptible	Intermediate	Resistant
Chloramphenicol	*Enterobacteriaceae* and *Staphylococcus*	C 30	30 μg	≥18	13–17	≤12
Ciprofloxacin	*Enterobacteriaceae* and *Staphylococcus*	CIP 5	5 μg	≥21	16–20	≤15
Penicillin	*Staphylococcus*	P 10	10 U	≥29		≤28
Streptomycin	*Enterobacteriaceae*	S 10	10 μg	≥15	12–14	≤11
Tetracycline	*Enterobacteriaceae* *Staphylococcus*	TE 30 TE 30	30 μg 30 μg	≥15 ≥19	12–14 15–18	≤11 ≤14
Trimethoprim	*Enterobacteriaceae* and *Staphylococcus*	TMP 5	5 μg	≥16	11–15	≤10

This table includes the antibiotics used in this exercise and contains data provided by the Clinical and Laboratory Standards Institute (CLSI). Permission to use portions (specifically Tables 2A and 2C) of M100-S24 (Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement) has been granted by CLSI. The interpretive data are valid only if the methodology in M02-A11 (Performance Standards for Antimicrobia Disk Susceptibility Tests—11th edition; Approved Standard) is followed. CLSI frequently updates the interpretive tables through new editions of the standard and supplements. Users should refer to the most recent editions. The current standard may be obtained from CLSI, 950 West Valley Road, Suite 2500, Wayne, PA 19087. Contact also may be made via the website (www.CLSI.org), email (customerservice@clsi.org) and by phone (1.877.447.1888).

7

✓ Materials

Per Student Group

- ☐ Two Mueller-Hinton agar plates
- ☐ Penicillin, chloramphenicol, tetracycline (optional), trimethoprim, streptomycin (optional), and ciprofloxacin antibiotic disks (and/or other disks as available)
- ☐ Antibiotic disk dispenser or forceps for placement of disks
- ☐ Screw-capped jar of alcohol (for sterilizing forceps, if used)
- ☐ Two sterile cotton swabs
- ☐ One metric ruler
- ☐ Sterile saline (0.85%)
- ☐ Sterile transfer pipettes
- ☐ One McFarland 0.5 standard with card
- ☐ Black, nonreflective poster board (8.5" × 11")
- ☐ Spectrophotometer (optional, in place of McFarland standard)
- ☐ Three matched, sterile, 1 cm cuvettes (optional, in place of McFarland standard)

- ☐ Three sterile transfer pipettes (optional, in place of McFarland standard)
- ☐ Two tryptic soy agar (or nutrient agar) plates (for optional procedure)
- ☐ Fresh broth cultures of:
  - *Escherichia coli*
  - *Staphylococcus aureus* (BSL-2)

Medium Recipe

Mueller-Hinton II Agar

• Beef extract	2.0 g
• Acid hydrolysate of casein	17.5 g
• Starch	1.5 g
• Agar	17.0 g
• Distilled or deionized water	1.0 L

pH 7.2–7.4 at 25°C

Procedure

Lab One

1. Gently mix the *E. coli* culture and the McFarland standard until they reach their maximum turbidity.
2. *Note*: If using a spectrophotometer, skip to step 4. Holding the culture and McFarland standard upright in front of you, place the card behind them so you can see the black line(s) through the liquid in the tubes. As you can see in Figure 7.5, the line becomes distorted by turbidity in the tubes. Use the black line to compare the turbidity level of the two tubes. Dilute the broth with sterile saline until it appears to have the same level of turbidity as the standard. If it is not turbid enough, incubate it until it reaches that level.
3. Repeat steps 1 and 2 with the *S. aureus* culture. **Use BSL-2 precautions when working with *S. aureus*.** Continue with step 7.
4. If preferred, a spectrophotometer (Appendix E) may be used to bring the samples to the proper density. Transfer uninoculated broth (the same as used to grow the *E. coli* and *S. aureus* cultures) into one of the cuvettes using a sterile transfer pipette. Use this as a blank.
5. Mix the *E. coli* culture and aseptically transfer some broth into the second cuvette. Properly dispose of the pipette, and then determine absorbance of the *E. coli* broth at 625 nm. Either dilute the culture or let it grow in order to bring its absorbance to 0.08 to 0.10. Use a sterile transfer pipette to return the sample to the culture tube.
6. Repeat step 5 with the *S. aureus* culture. **Use BSL-2 precautions in transferring it.** When the two broths have the correct turbidity, continue with step 7.
7. Dip a sterile swab into the *E. coli* broth. As you remove it, press and rotate the cotton tip against the side of the tube to remove excess broth.
8. Inoculate a Mueller-Hinton plate with *E. coli* by streaking the entire surface of the agar three times with the swab. Your goal is confluent growth, so make the streaks right next to each other. When you have covered the surface, rotate the plate 1/3 turn and repeat the streaking of the inoculum already on the plate, using the same technique to produce confluent growth. Then rotate the plate another 1/3 turn and repeat. Properly dispose of the swab and replace the plate's lid.
9. Using a fresh sterile swab, inoculate the other plate with *S. aureus* in the same fashion to produce confluent growth. **Use BSL-2 precautions and properly dispose of the swab.** Replace the plate's lid.
10. Label the plates with the organisms' names, your name, and the date.
11. Apply the penicillin, chloramphenicol, ciprofloxacin, and trimethoprim disks to the agar surface of each plate. You can apply the disks singly using alcohol-flamed forceps[1]. Be sure to space the disks sufficiently (4 cm to 5 cm) to prevent overlapping zones of inhibition and keep them away from the edge. Gently press the disks onto the agar with the forceps so they make good contact. ***Caution***: Keep the flaming forceps away from the alcohol jar. If the alcohol in the jar catches fire, extinguish it by replacing its lid. Continue with step 13.
12. Alternatively, you can use a disk dispenser to deliver the disks (Fig. 7.6). If the dispenser is self-tamping, continue with step 13. If not, use the alcohol-flamed forceps to gently press each disk so it makes full contact with the agar. Continue with step 13.
13. Tape the plates closed around the edges. Invert them and incubate aerobically at 35 ± 2°C for 16 to 18 hours. Have a volunteer in the group remove and refrigerate the plates at the appropriate time if this does not match your lab schedule.

[1] Keep the forceps in the alcohol jar until ready to use them. Then, close them as you remove them from the alcohol. This will trap some alcohol between the tips. Pass the forceps *through* the flame and allow the alcohol to burn off *away from the alcohol jar and the flame*. When the flame burns out, give the forceps a few seconds to cool, and then place the disk on the plate. Return the forceps to the alcohol jar until needed.

Lab Two

1. Remove the plates from the incubator (or refrigerator). Hold the plate over the black, nonreflective poster board and examine the plate with reflected light. The edge of a zone is where no growth is visible to the naked eye. Measure the diameter of each zone of inhibition in millimeters (Fig. 7.7).
2. Using Table 7-4 and those provided with your antibiotic disks (if you used additional antibiotics), record your results in the table provided on the data sheet, page 535.

Optional Procedure Beginning with Lab Two

1. Obtain two TSA or NA plates. With your marking pen, divide the plates into four sectors (or more if you used more antibiotics).
2. Label each sector with an antibiotic. It is easiest if you label in the same order as they are found on the MH plates.
3. Label one plate *E. coli* and the other *S. aureus*.
4. Using a sterile loop for each transfer, obtain a sample from each antibiotic's zone of inhibition on the *E. coli* plate, and inoculate the corresponding sector on the TSA (or NA) plate. If there is no zone for a particular antibiotic, no transfer is necessary.
5. Repeat with the *S. aureus* MH plate. Use a sterile wooden stick or disposable loop for these transfers and dispose of them promptly and properly after each. **Use BSL-2 precautions when working with *S. aureus*.**
6. Incubate the plates aerobically at 35 ± 2° for 24–48 hours.

Lab Three

1. Remove the plates from the incubator and examine each sector for growth.
2. Record your observations and answer the questions on the data sheet.

References

Clinical Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard*, 11th ed. CLSI document M02–A11. Wayne, PA: CLSI, 2012.

Clinical Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Fourth Informational Supplement*. CLSI document M100–S24. Wayne, PA: CLSI, 2014.

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 128 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Ferraro, Mary Jane, and James H. Jorgensen. Chap. 15 in *Manual of Clinical Microbiology*, 8th ed. Patrick R. Murray, Ellen Jo Baron, James. H. Jorgensen, Michael A. Pfaller, and Robert H. Yolken, eds. Washington, DC: ASM Press, 2003.

Mims, Cedric, Hazel M. Dockrell, Richard V. Goering, Ivan Roitt, Derek Wakelin, and Mark Zuckerman. Chap. 33 in *Medical Microbiology*, 3rd ed. Philadelphia: Mosby, 2004.

Tille, Patricia M. 2014. Pages168–170 and 174–178 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Winn, Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckenberger, and Gail Woods. Pages 975 and 983–989 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Philadelphia: Lippincott Williams & Wilkins, 2006.

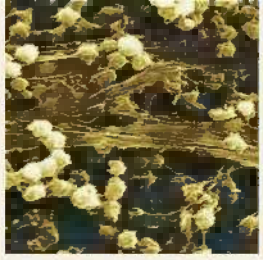
Zimbro, Mary Jo, and David A. Power, eds. Page 376 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Company, 2003.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-3

Antimicrobial Susceptibility Test: Disk Diffusion (Kirby-Bauer) Method

OBSERVATIONS AND INTERPRETATIONS

1 Record the zone diameters in mm in Table 1. Using Table 7-4, page 531, as a guide, enter "S" if the organism is susceptible and "R" if it is resistant to the antibiotic. (Not all combinations of antibiotics and organisms are available.)

Table 1: Interpretation of Zone Diameters

Organism	Chloramphenicol		Ciprofloxican		Trimethoprim		Penicillin	
	Zone Diameter	S/R	Zone Diameter	S/R	Zone Diameter	S/R	Zone Diameter	S/R
E. coli								
S. aureus								

2 If you did the optional exercise, record "G" for growth and "NG" for no growth by cells recovered from the zone of inhibition on the TSA/NA plates in Table 2. Then, decide and record if the antibiotic is bactericidal or bacteriostatic.

Table 2: Interpretation of Zone Diameters

Antibiotic	Chloramphenicol		Ciprofloxican		Trimethoprim		Penicillin	
	NG/G	-static or -cidal	NG/G	-static or -cidal	NG/G	-static or -cidal	NG/G	-static or -cidal
E. coli								
S. aureus								

QUESTIONS

1 *All aspects of the Kirby-Bauer test are standardized to assure reliability.*

a. *What might be the consequence of pouring the plates 2 mm deep instead of 4 mm deep?*

b. *The Mueller-Hinton II plates are supposed to be used within a specific time after their preparation and should be free of visible moisture. What negative effect(s) might moisture have on the test?*

2 *In clinical applications of the Kirby-Bauer test, diluted cultures (for the McFarland standard comparison) must be used within 30 minutes. Why is this important?*

3 E. coli *and* S. aureus *were chosen to represent Gram-negative and Gram-positive bacteria, respectively.*

a. *For a given antibiotic, is there a difference in susceptibility between the Gram-positive and Gram-negative bacteria?*

b. *If so, what difference(s) do you see?*

c. *Do these agree with the data in Table 7-4?*

4 *How does the antibiotic get from the disk into the agar?*

5 *Does the agar have antibiotic beyond the zone of inhibition? How does your answer tie in with MIC?*

6 *Suppose you do this test on a hypothetical* Staphylococcus *species with the antibiotics penicillin (P 10) and chloramphenicol (C 30). You record zone diameters of 25 mm for the chloramphenicol and penicillin disks. Which antibiotic would be more effective against this organism? What does this tell you about comparing zone diameters to each other and the importance of the zone diameter interpretive table?*

7 *In the optional exercise, what was the purpose of inoculating TSA (or NA) plates with samples taken from the zones of inhibition?*

EXERCISE 7-4

Clinical Biofilms

Theory

According to Elvers and Lappin-Scott (2004), a biofilm is a "complex association or matrix of microorganisms and microbial products attached to a surface." Development begins with formation of a **conditioning film** composed of biomolecules and particles from the environment on the surface. This results in the surface becoming more hydrophilic and negatively charged. Following this comes attachment of **planktonic** microorganisms to the surface. The precise mechanism varies depending on the amount of fluid movement and other factors; however, biofilm formation invariably depends upon the microbial community itself through secretion of an extracellular polysaccharide (**glycocalyx**). As more microbes become attached, they change the composition of the biofilm's chemistry, providing a suitable environment for still other microorganisms. Eventually, the biofilm gets thick enough that detachment (which can be the result of erosion, abrasion, or simple breakage) becomes a factor. At some point a steady state is reached in which addition to the biofilm is compensated by loss from the biofilm.

Biofilms occur in natural environments, but also are formed in industrial and medical settings. It is the medical with which this lab is concerned. Indwelling devices, such as needles and catheters, are common locations for biofilm development. Reduced susceptibility of the biofilm community to antimicrobics (by a factor of 100–1,000 times compared to their planktonic counterparts!) along with their natural ability to detach make these biofilms problematic in the production of **health-care associated (nosocomial)** infections (HAIs). A 2011 survey of 183 U.S. hospitals showed that 4% of the 11,290 patients developed one or more HAIs, with slightly over 25% associated with catheters and other devices. *S. aureus* was the second-most common pathogen associated with HAIs, second only to *Clostridium difficile* (Magill et al. 2014).

Application

Staphylococcus aureus and *S. epidermidis* are notorious for forming biofilms on invasive medical devices, resulting in nosocomial infections.

In This Exercise

You will grow a mixed culture of *Staphylococcus aureus* and *S. epidermidis* for one week in a test tube, stain it, and observe for evidence of a biofilm.

Materials

Per Student Group

- ☐ Overnight broth cultures of *Staphylococcus aureus* (BSL-2) and *S. epidermidis*
- ☐ Two tubes of TSB enriched with 1% glucose
- ☐ Phosphate buffered saline (PBS), pH 7.3
- ☐ 0.1% crystal violet
- ☐ Deionized water bottle
- ☐ Test tube rack
- ☐ Receptacle for culture media disposal

Medium, Stain, and Reagent Recipes

Tryptic Soy Broth Plus 1% Glucose

• Tryptic soy broth	1.0 L
• Glucose	10.0 g

Crystal Violet Stain (0.1%)

• Gram crystal violet	0.1 mL
• dH_2O	99.9 mL

10× Phosphate Buffered Saline (PBS)

• Na_2HPO_4, anhydrous, reagent grade	12.36 g
• $NaH_2PO_4 \cdot H_2O$, reagent grade	1.80 g
• NaCl, reagent grade	85.00 g
• Dissolve ingredients in distilled water to a final volume of 1 L	

PBS Working Solution (0.01 M Phosphate, pH 7.6)

• Stock solution	100 mL
• dH_2O	900 mL

Procedure

Lab One

1. Heavily inoculate one TSB + 1% glucose tube with both *Staphylococcus aureus* and *S. epidermidis*. Use **BSL-2 precautions when transferring *S. aureus*.** Label this tube with the names of the organisms.
2. Label the second tube "negative control" and do not inoculate it.
3. Incubate the tubes for 24–48 hours at 35 ± 2°C.

Lab Two

1. Place the disposal receptacle on a disinfectant-soaked towel. Then, carefully pour the broth from each tube into the receptacle. Be sure not to spill or drip culture when pouring. If you do spill or drip culture, clean it up with your lab's disinfectant and wash your hands with your lab's antiseptic.
2. Gently rinse each tube with PBS twice and pour it into the receptacle designated for disposal.
3. Air-dry the tubes in an inverted position on a test tube rack.
4. Stain both tubes with 0.1% crystal violet solution for 10 minutes at room temperature. Be sure to add more stain than the original volume of broth in the tubes.
5. Gently wash the tubes with dH_2O to remove unbound crystal violet.
6. Air-dry the tubes in an inverted position on a test tube rack.
7. Compare your results with Figure 7.8. You are looking for a purple film adhering to the inside of the experimental tube. Ignore any dark ring at the surface. Record your observations and answer the questions on the data sheet.
8. Wipe down the test tube rack with disinfectant.

7.8 Staphylococcal Biofilm ♦ The faint purple is the biofilm stained with dilute crystal violet. The tube on the right is an uninoculated control.

7

References

Elvers, Karen T. and Hilary M. Lappin-Scott. Biofilms and Biofouling, Chap. 12 in *The Desk Encyclopedia of Microbiology*. Moselio Schaechter, ed. San Diego: Elsevier Academic Press, 2004.

Hirshfield, Irvin N., Subit Barua, and Paramita Basu. Overview of Biofilms and Some Key methods for Their Study, Chap. 42 in *Practical Handbook of Microbiology*, 2nd ed. Emanuel Goldman and Lorrence H. Green, eds. Boca Raton, FL: CRC Press, Taylor and Francis Group, 2009.

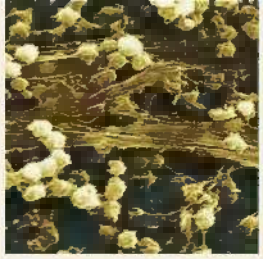
Magill, Shelley S., Jonathan R. Edwards, Wendy Bamberg, et.al, for the Emerging Infections Program Healthcare-Associated Infections and Antimicrobial Use Prevalence Survey Team. *N Engl J Med* 370 (2014): 1198–1208. Published electronically March 27, 2014: doi: 10.1056/NEJMoa1306801.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-4

Clinical Biofilms

OBSERVATIONS AND INTERPRETATIONS

1 Record your results for the biofilm test in the table.

Tube	Biofilm (yes/no)
Control	
Staphylococcus mixture	

QUESTIONS

1 *What is the role of the control tube? Be specific.*

2 *Why were* Staphylococcus aureus *and* S. epidermidis *chosen as experimental organisms?*

3 *Define "nosocomial infection."*

4 *What two factors make a biofilm in an intravenous line especially dangerous to the patient?*

Morbidity and Mortality Weekly Report (MMWR) Assignment

EXERCISE 7-5

Theory

Epidemiology is the study of the causes, occurrence, transmission, distribution, and prevention of diseases in a population. The Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, is the national clearinghouse for epidemiological data (Fig. 7.9). The CDC receives reports related to the occurrence of 30 notifiable diseases (Table 7-5) from the United States and its territories, and compiles the data into tabular form, available in the publication *Morbidity and Mortality Weekly Report* (MMWR).

Two important disease measures that **epidemiologists** collect are **morbidity** (sickness) and **mortality** (death). Morbidity relative to a specific disease is the number of susceptible people who have the disease within a defined population during a specific time period. It usually is expressed as a rate. Because population size fluctuates constantly, it is conventional to use the population size at the midpoint of the study period. Also, the units for the rate fraction are "cases per person" and usually are small decimal fractions. To make the calculated rate more "user-friendly," it is multiplied by some power of 10 ("K") to achieve a value that is a whole number. Thus, a morbidity rate of 0.00002 is multiplied by 100,000 (10^5) so it can be reported as 2 cases per 100,000 people rather than 0.00002 cases per person. Morbidity rate is calculated using the following equation:

$$\text{Morbidity Rate} = \frac{\text{number of existing cases in a time period}}{\text{size of at-risk population at midpoint of time period}} \times \text{K}$$

Mortality, also expressed as a rate, is the number of people who die from a specific disease out of the total population afflicted with that disease in a specified time period. It, too, is multiplied by a factor "K" so the rate can be reported as a whole number of cases. The equation is:

$$\text{Mortality Rate} = \frac{\text{number of deaths due to a disease in a time period}}{\text{number of people with that disease in the time period}} \times \text{K}$$

Minimally, an epidemiological study evaluates morbidity or mortality data in terms of **person** (age, sex, race, etc.), **place**, and **time**. Sophisticated analyses require training in biostatistics, but the simple epidemiological calculation you will be doing can be performed with little mathematical background. You will calculate **incidence rate**, which is the occurrence of new cases of a disease within a defined population during a specific period of time. As before, "K" is some power of 10 so the rate can be reported as a whole number of cases.

7.9 Centers for Disease Control ◆ Shown is the entrance to the CDC campus in Atlanta, Georgia.

$$\text{Incidence Rate} = \frac{\text{number of new cases in a time period}}{\text{size of at-risk populations at midpoint of time period}} \times \text{K}$$

Because our focus is microbiology, we will deal only with **infectious diseases**—those caused by biological agents such as bacteria and viruses. **Noninfectious diseases**, such as stroke, heart disease, and emphysema, also are studied by epidemiologists but are not within the scope of microbiology.

Application

An understanding of the causes and distribution of diseases in a population is useful to health-care providers in a couple of ways. First, awareness of what diseases are prevalent during a certain period of time aids in diagnosis. Second, an understanding of the disease, its causes, and transmission can be useful in implementing strategies for preventing it.

In This Exercise

First you will choose a disease from the list of notifiable diseases in Table 7-5. Then you will collect data for the United States over the past two complete years. Once you have collected data, you will construct graphs illustrating the cumulative totals and incidence values for each year.

✓ Materials

A computer with Internet access or access to printed copies of *Morbidity and Mortality Weekly Report*.

Procedure

1. Choose a disease from Table 7-5 and record its name on the data sheet, page 546.
2. Go to the CDC website (http://www.cdc.gov). Then follow these links. (*Note:* Websites often are revised, so if the site does not match this description exactly, it still is probably close. Improvise, and you will find what you need. These links are current as of November 2014):
   - Click on Morbidity and Mortality Weekly Report (MMWR) near the bottom left of the home page under "About CDC" and "Science."
   - Click on "Publications" in the menu bar at the left. This will drop down a new menu.
   - Click on "Weekly Report."
   - Click on "Past Volumes (1982–201X)."
   - Click on the link to the volume for the most recent complete year. For example, if it is 2015, then the most recent complete year would be 2014. It might look something like this: "Volume 63 (2014) Weekly *MMWR* Issues."[1]
   - Scroll to the bottom of this new page and you will find the first issue for that volume. Then click on the link to "Notifiable Diseases and Mortality Tables."
   - On this new page, click on the link to "Table II" and you are where you need to be![2]
   - Table II in 2014 was divided into 11 parts, but that often changes from year-to-year, so be flexible. The diseases are listed alphabetically. Find the disease you have chosen and write the *cumulative* number of cases in the United States for Week 1 of the two years you are studying. For instance, if you are studying 2014 and 2013, you would take the numbers from the columns titled *Cum 2014* and *Cum 2013*. Record these on the data sheet. Using the years 2014 and 2013 as examples, you will find that the 2013 number reported for a particular week in the 2014 table might differ from the reported number in the same week of the 2013 table. This is a result of corrections in reported 2013 numbers in the 2014 table. Don't fret. This is out of your control! Just record the numbers as given.
   - Return to the page with the links to all the issues from the latest year you are pulling information from. Scroll down to Week 2 and click on the link to "Notifiable Diseases and Mortality Tables" and get the data for the last two complete years as before. Repeat this process for each week through *MMWR* Week 52. (Alternatively, you can just change the week number in the URL and press return.[3]) As you go, record the cumulative totals on the data sheet.
3. Answer the questions and complete the activities on the data sheet.
4. At some point before the assignment is due, go to the National Notifiable Diseases Surveillance System (NNDSS) page (http://wwwn.cdc.gov/nndss/) and read the information on the home page. Then, click the link to "Data Collection and Reporting" to see how the data you are using has been collected.

[1] Volumes for the next few years are: Volume 63, 2014; Volume 64, 2015; Volume 65, 2016; Volume 66, 2017; and Volume 67, 2018.

[2] Table I lists the occurrence of infrequently reported notifiable diseases (< 1,000 cases in the previous year). Your instructor may allow you to choose selected diseases from this table in addition to the ones listed in Table II.

[3] The URL looks like this: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6340md.htm?s_cid=mm6340md_w. The numbers highlighted in bold are the week number, preceded by the volume number. So, 6340 means Volume 63, Week 40. Change the 40 to 41, press return and you should end up on Table II, Week 41. Do not change the second set of numbers because that doesn't work. Thanks to former student Joe Montes, Jr. for this shortcut tip!

TABLE 7-5 Notifiable Diseases in the United States and Its Territories Posted in Table II of MMWR (as of November 2014). ♦
Not all diseases have a high incidence, but do not be misled. Smaller numbers won't necessarily make your work any easier! The only diseases unsuitable for this assignment are **Invasive Pneumococcal Disease** (<5) and **Rabies, animal**, because you won't have a population size to use for your calculations.

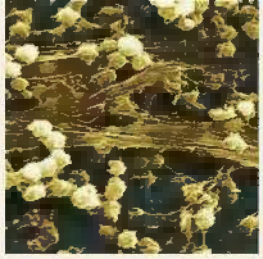
Selected Notifiable Diseases	
Babesiosis	Legionellosis
Chlamydia trachomatis infection	Lyme Disease
Coccidioidomycosis	Malaria
Cryptosporidiosis	Meningococcal Disease
Dengue Fever	Mumps
Ehrlichiosis chaffeensis	Pertussis
Anaplasma phagocytophilum	**Rabies, animal**
Girardiasis	Salmonellosis
Gonorrhea	Shiga toxin-producing *E. coli* (STEC)
Haemophilus influenzae, invasive, all ages and serotypes	Shigellosis
Hepatitis A	Spotted Fever Rickettsiosis–confirmed
Hepatitis B	Syphilis, primary and secondary
Hepatitis C	Varicella (chickenpox)
Invasive Pneumococcal Disease (all ages)	West Nile Virus—neuroinvasive
Invasive Pneumococcal Disease (<5)	West Nile Virus—non-neuroinvasive

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-5

Morbidity and Mortality Weekly Report (MMWR) Assignment

DATA AND CALCULATIONS

1 Go to the MMWR Morbidity Tables at the CDC website (www.cdc.gov). Record the cumulative weekly totals for your selected disease for the last two complete years in the data table on the following pages. No calculation is necessary regarding these numbers at this point. Because they are cumulative totals, they should be greater than or equal to the preceding week. Nevertheless, because the reported numbers are provisional and subject to change, this may not always be the case. Simply record the numbers from the CDC website as they are reported.

2 Calculate the number of new cases during each 4-week period (weeks 1–4, weeks 5–8, etc.) for both years by subtracting the total at the end of a 4-week period from the total at the end of the next 4-week period. That is, for the second 4 weeks subtract the total at the end of week 4 from the total at the end of week 8. Record these in the table on page 546 in the column labeled "4-Week Totals." Show a sample calculation for the third 4-week total in the space below.

3 Calculate national incidence values of each 4-week period during the two years using the calculated 4-week totals (weeks 1–4, weeks 5–8, etc.) in the numerator. Use the U.S. population size on July 1 in the denominator. This value can be obtained from the U.S. Census Bureau website at http://www.census.gov/popest/, and then clicking on "Current Estimates Data" under "Quick Links." On the new page, you will see a table with three columns. The first row is for the Nation, Total Population. In the right column on this row there is a link (e.g., V2013). Click on it and on the new page you will see a link for "Monthly Population Estimates…" Click on this and find the population for July of the two years you are reporting on and record these below. Be sure to choose an appropriate value for K, and do not abuse significant figures! Show a sample calculation for the third 4-week period of the most recent year in the space below.

Estimated population size for the year 20 ____ :

$$\text{Incidence Rate} = \frac{\text{number of new cases in a time period}}{\text{size of at-risk population at midpoint of time period}} \times K$$

Morbidity Data for _____ during the years 20____ and 20____

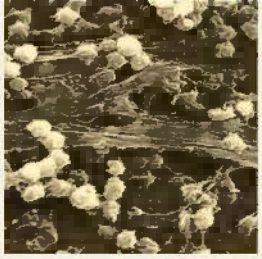
Week	Cumulative Totals by Week Year (20__)	4-Week Totals Year (20__)	Incidence Values Year (20__)	Cumulative Totals by Week Year (20__)	4-Week Totals Year (20__)	Incidence Values Year (20__)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						
22						
23						
24						

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-5

(continued)

Week	Cumulative Totals by Week Year (20)	4-Week Totals Year (20)	Incidence Values Year (20)	Cumulative Totals by Week Year (20)	4-Week Totals Year (20)	Incidence Values Year (20)
25						
26						
27						
28						
29						
30						
31						
32						
33						
34						
35						
36						
37						
38						
39						
40						
41						
42						
43						
44						
45						
46						
47						
48						
49						
50						
51						
52						

QUESTIONS

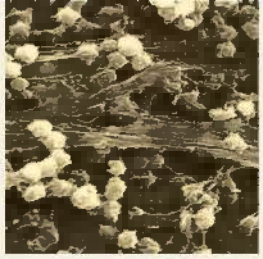
1 *Characterize the disease you have chosen. Be sure to include causative agent (by scientific name), symptoms, mode of transmission, and treatment. Cite your references.*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-5

(continued)

2 *Prepare a graph that illustrates the cumulative data for each week over the two years. Be sure to include a title, axis labels with appropriate units, and legends in your graph.*

3 *Graph the calculated national incidence values over the two years studied. In your graph, be sure to include a title, axis labels with appropriate units, and legends.*

4 *Briefly compare national trends of your chosen disease for the two years. Are the number of cases similar? Does the incidence appear to be seasonal?*

EXERCISE 7-6

Epidemic Simulation

Theory

As mentioned in Exercise 7-5, epidemiology is the study of the causes, occurrence, transmission, distribution, and prevention of diseases in a population. Infectious diseases are transmitted by ingestion, inhalation, direct skin contact, open wounds or lesions in the skin, animal bites, direct blood-to-blood contact as in blood transfusions, and sexual contact. Infectious diseases can be transmitted by way of sick people or animals, healthy people or animals carrying the infectious organism or virus, water contaminated with human or animal feces, contaminated objects (**fomites**), aerosols, or biting insects (**vectors**).

When a disease is transmitted from an area such as the heating or cooling system of a building or from contaminated water that infects many people at once, it is called a **common source epidemic**. **Propagated transmission** is a disease transmitted from person to person. The first case of such a disease is called the **index case**. Determining the index case is the object of today's lab exercise.

A second objective of today's lab is to gather data to be used in performing simple epidemiological calculations. You will be calculating **incidence** and **prevalence rates** for the "disease" spreading through your class.

Incidence rate is the number of new cases of a disease reported in a defined population during a specific period of time. Incidence rate is calculated by the following equation:

$$\text{Incidence rate} = \frac{\text{number of new cases in a time period}}{\text{size of at-risk population at midpoint of time period}} \times K$$

Because population size fluctuates constantly, it is conventional to use the population size at the midpoint of the study period. Also, the usually small decimal fraction obtained for the rate is multiplied by some power of 10 (K in the equation) to get its value up to a number bigger than one. That is, an incidence rate of 0.00002 is multiplied by 100,000 (10^5) so it can be reported as two cases per 100,000 people, rather than 0.00002 cases per person.

You also will be calculating one form of prevalence rate called **point prevalence**, the number of cases of a disease at a specific point in time in a defined population. It is calculated by the following equation:

$$\text{Point prevalence} = \frac{\text{number of existing cases at a point in time}}{\text{size of at-risk population}} \times K$$

As with incidence rate, the calculated prevalence is multiplied by some power of 10 (K in the equation) to bring the number up to one or more.

Application

Epidemiologists have the task of identifying the source of a disease and establishing the mode of its transmission. Further, epidemiologists characterize diseases quantitatively, using measures such as incidence and prevalence. Hopefully, this information will allow them to make recommendations for prevention.

In This Exercise

One of you will become the "index case" in a simulation of an epidemic, and many of you who contact this person directly and indirectly will become "infected." Your job, besides having a little fun, will be to collect the data and determine which one of you is the index case. You will also use the data to calculate incidence and prevalence rates for your class over a hypothetical time period.

7

✓ Materials

Per Student

- ☐ Examination glove (latex or synthetic)
- ☐ Lab coat
- ☐ Numbered Petri dish containing a piece of hard candy (one will be contaminated with *Serratia marcescens*[1] or other microbe as chosen by your instructor; the others will be moistened with water)[2]
- ☐ One nutrient agar plate
- ☐ Two sterile cotton applicators and sterile saline
- ☐ Marking pen

[1] *S. marcescens* was chosen because of its obvious color. Other less-distinctive organisms can be substituted, if desired.

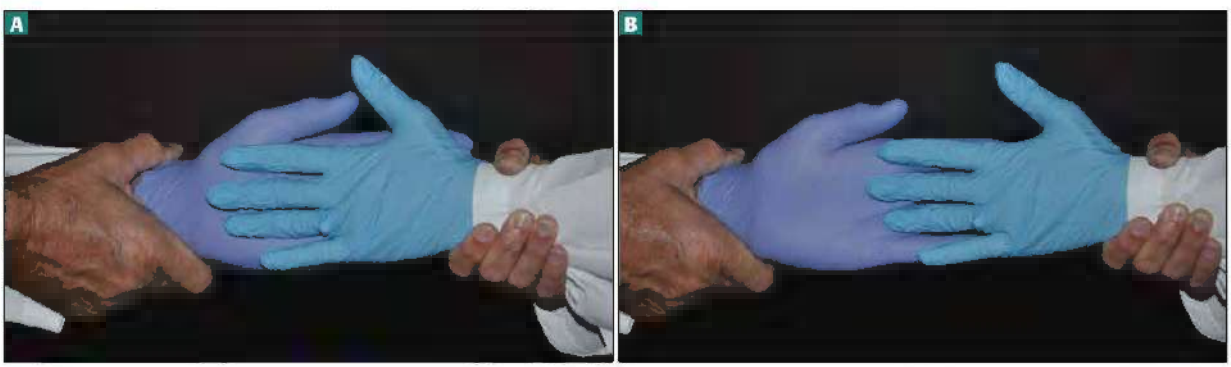
[2] Petri dishes should be numbered according to the number of students in the class. There should be no gaps in the sequence.

Procedure

Lab One

1. You must follow the instructions precisely. Your instructor may want the class to do each step at the same time, and if so, do not get ahead. This is being done for safety. Containers for swab and glove disposal should be located around the room so all students have easy access. You should also look at Figure 7.10 to see how to separate gloves safely.
2. Obtain a Petri dish containing candy, two sterile cotton applicators, a tube of sterile saline, and a nutrient agar plate. Record the number of your candy dish here and circle that number in the "case" column of the data sheet, page 555. Then, put away all your papers and books for the duration.

   ☐
3. Put on your lab coat.
4. Mark a line on the bottom of the agar plate to divide it into two halves. Label the sides "1" and "2." Also write your name and the number of your candy dish on your NA plate.
5. Open a package of sterile, cotton-tipped applicators so they can be easily removed. They are usually packaged in pairs, and that is all you need.
6. Put a glove on your nondominant hand.
7. Using your ungloved hand, remove one cotton applicator, moisten it in the sterile saline, and sample the palm of your gloved hand. Be sure not to touch anything else with the palm of the glove. When finished, zigzag inoculate side 1 of the plate with the cotton applicator (Fig. 1.37). Discard the applicator in an appropriate autoclave container.
8. Pick up the candy with your gloved hand and roll it around until a good amount of it is transferred to the glove. Drop the candy back into the Petri dish, close it with your ungloved hand, and touch nothing with your gloved hand. Just sit with your elbow on the table and your gloved hand in the air. All but one student in the class just has messy candy on their glove at this point, but you might be the one with *S. marcescens*. Be mindful of this.
9. **Read this entire step before doing anything!** Students ("cases") are identified by their candy dish number. Using gloved hands only, case #1 rubs hands with case #2, making sure to transfer anything that may be on the palm of the glove to the palm of the other student's glove. Both students must hold on to the cuff of their glove throughout this step so it doesn't come off. After a few seconds of rubbing, they should *gently slide their hands apart, as shown in Figure 7.10*. They must not "snap" the gloves as they separate because this will produce aerosols. Case #1 should go back to his or her desk and sit with the elbow of their gloved hand on the table and their hand in the air.
10. Then case #2 rubs gloved hands with #3, and #3 with #4, and so on until all students have contacted gloved hands. Continue to be careful not to snap the gloves when you separate. Hold on to the cuff when transferring and separating to prevent the glove from coming off your hand.
11. When all students have rubbed hands, the last (highest numbered) case should rub hands with case #1.
12. With a second sterile applicator, sample the palm of your gloved hand as before and inoculate side 2 of the plate. Be sure to sample the area of the glove that contacted other students' gloves. Dispose of the cotton applicator in the nearest appropriate



7.10 Sliding the Gloves Apart ◆ After rubbing palms of the hands, students should separate by gently sliding their gloved hands apart (as in **A** and **B**). Be sure to hold on to the glove's cuff to prevent it from coming off. These safety precautions must be followed and are designed to prevent the gloves from snapping apart (they will be sticky) and producing aerosols.

autoclave container. Do not walk across the room with the swab. If necessary, put it into a can, tip down until the exercise is completed.

13 Remove your glove by inserting the thumb of your other hand under the cuff and rolling it off your hand, inverting it in the process. Dispose of it properly.

14 Wash your hands thoroughly with antiseptic soap, if available.

15 Discard all materials (except your inoculated plate) in appropriate containers. Be sure to tape the lid on your candy dish before you dispose of it.

16 Wipe down your work area with disinfectant.

17 Tape the lid on your inoculated plate. You may find it convenient to tape all plates from your group in a single stack so they can be retrieved more easily.

18 Invert the inoculated plate and incubate it at 25°C for 24 to 48 hours.

Lab Two

1 Remove the plates from the incubator, and examine them for characteristic reddish growth of *Serratia marcescens*.

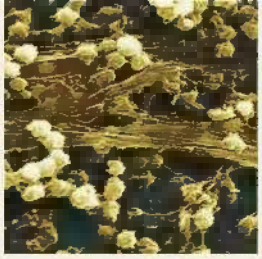
2 Enter your results on the data sheet and follow the instructions for establishing the index case, incidence rate, and point prevalence.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-6

Epidemic Simulation

DATA AND CALCULATIONS

1 On the following table, circle the case number that matches your candy dish number.

2 After the plates have been incubated, compile data for your class population (each case is the student's candy number in the exercise). The organism spread during contact between student pairs was *Serratia marcescens*, which produces reddish-orange colonies. You will likely see growth on both sides of the plate, but red colonies are the only sign of disease. If there is reddish-orange growth on side 2 of the plate, consider the person to have the disease and record a "+" in the appropriate box. If there is no red growth on the plate or there is no difference between the two sides, consider the person to be healthy and record a "–" in the appropriate box.

Case	Week	Disease (+/–)
1	1	
2	1	
3	1	
4	1	
5	2	
6	2	
7	2	
8	2	
9	2	
10	3	
11	3	
12	3	
13	3	
14	3	
15	3	
16	4	
17	4	
18	4	
19	4	
20	4	

Case	Week	Disease (+/–)
21	4	
22	4	
23	5	
24	5	
25	5	
26	5	
27	5	
28	6	
29	6	
30	6	
31	7	
32	7	
33	7	
34	7	
35	8	
36	8	
37	8	
38	9	
39	9	
40	10	

3 Identify the case number of the index patient.

4 Use the data obtained to calculate incidence and prevalence rates in your class population. For this purpose, assume that the cases have been identified over a 10-week period (or a shorter period, depending on how many students participated in the simulation). Also assume that the duration of the disease is 7 days, so new cases in 1 week are still diseased in the next week but are healthy by the following week.

Record the population size: _____

Record incidence and point prevalence rates for each week in the table.

Week	Incidence	Prevalence
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

QUESTIONS

1 *Explain how you determined the index case.*

2 *What was the purpose of side 1 on each plate?*

3 *Suggest possible reasons (based on the execution of the simulation) for cases showing no* S. marcescens *growth between the cases that exhibited growth.*

4 *Suggest how the gaps could represent "subclinical infections" or "carriers" in this simulation.*

5 *Within the context of this simulation, how could you characterize any "non-* Serratia marcescens *growth on side 1 or 2 of your plate?* (**Hint:** *"Contamination" is not an adequate answer!*)

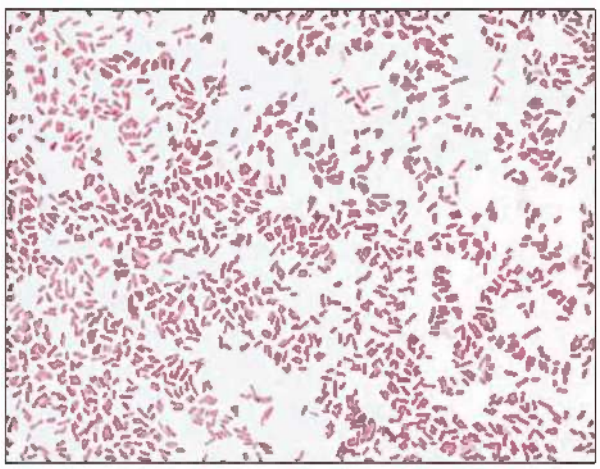
Identification of *Enterobacteriaceae*

EXERCISE 7-7

Theory

The *Enterobacteriaceae* (Fig. 7.11) comprise a diverse family of Gram-negative rods that inhabit the human intestinal tract. The family has grown from 12 genera and 36 species in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (1974) to 44 genera and 176 species in *Bergey's Manual of Systematic Bacteriology*, 2nd ed., Volume 2, *Part B–The Gammaproteobacteria* (2005), with the expectation that these numbers will increase as more is learned. This expansion has brought additional variability into the group and the recognition that the human intestines are not their main habitat. *Enterobacteriaceae* are also common in the environment (soil) and have hosts as diverse as plants and insects. Still, the following major characteristics are true for the majority of species:

- Growth on MacConkey agar
- Gram-negative rods
- Facultative anaerobes (respiratory and fermentative metabolism)
- Oxidase negative
- Acid production from glucose (with or without gas)
- Most are catalase positive
- Most reduce NO_3 to NO_2
- Peritrichous flagella, if motile



7.11 *Escherichia coli*, a Common Species in the Family *Enterobacteriaceae* ◆ All *Enterobacteriaceae* are Gram-negative, oxidase-negative rods. They also ferment glucose to acid end products.

Many enterics are human pathogens (e.g., *Escherichia coli* 0157:H7—bloody diarrhea; *Klebsiella pneumoniae*—pneumonia of various types; *Proteus mirabilis*—urinary tract infections; *Salmonella typhi*—typhoid fever; *Shigella dysenteriae*—bacillary dysentery; and *Yersinia pestis*—plague) but just as many are harmless gut **commensals** or **opportunists.** The organisms for which the identification flowcharts in this exercise work are listed in Figure 7.12, and some are commonly associated with human infections. Your instructor will choose enteric unknowns appropriate to your microbiology course and facilities.

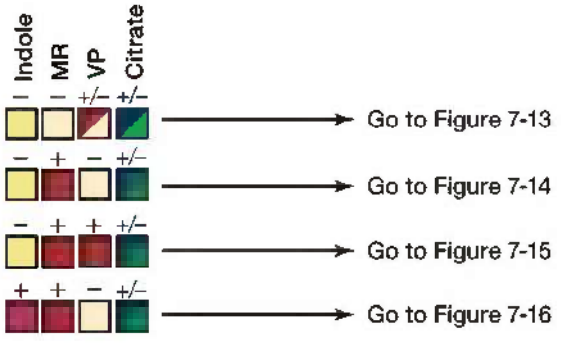
List of *Enterobacteriaceae* in Identification Charts and IMViC Results

*Citrobacter freundii**
Citrobacter koseri
Enterobacter aerogenes
Enterobacter cloacae
Escherichia coli
*Hafnia alvei**
Klebsiella pneumoniae+ (BSL-2)
Morganella morganii (BSL-2)
*Proteus mirabilis** (BSL-2)
Proteus vulgaris (BSL-2)
Providencia alcalifaciens
Providencia stuartii
Salmonella enterica++ (BSL-2)
Serratia liquefaciens
*Serratia marcescens**
*Shigella flexneri (Group B)** (BSL-2)
Shigella sonnei (Group D) (BSL-2)

*Organism shows up in more than one figure due to variable results of one or more IMViC tests

+*K. pneumoniae* is *K. pneumonie* subsp. *K. pneumoniae*

++*Salmonella enterica* is *Salmonella enterica* subsp. *enterica* and includes *S. enteritidis* and *S. typhimurium*



7.12 IMViC Results of *Enterobacteriaceae* ◆ The organisms listed are *Enterobacteriaceae* used in this exercise. Match the IMViC results of your isolate with one of the four shown, and then proceed with the flowchart in the figure indicated. Some organisms are listed in more than one flowchart because of variable results for a particular test. A box divided diagonally means either result for that test is a match. (Most results after Farmer et al., 2007.) ***Note:*** Each flowchart can be used to identify only the organisms listed.

Application

Identification of enterics from human specimens using traditional methods requires a coordinated and integrated use of biochemical tests and stains. Although several multi-test systems that utilize computer databases are available (see Exercises 5-29 and 5-30 for two examples), flowcharts are still a useful way to visualize the process of identification by elimination. It is striking how often just a few test results, when taken in combination, are necessary to identify an organism.

In This Exercise

This exercise will span several lab periods. You will be assigned an unknown enteric and run biochemical tests as directed by the flowcharts in Figures 7.13 through 7.16 to identify it.

✓ Materials

Per Class

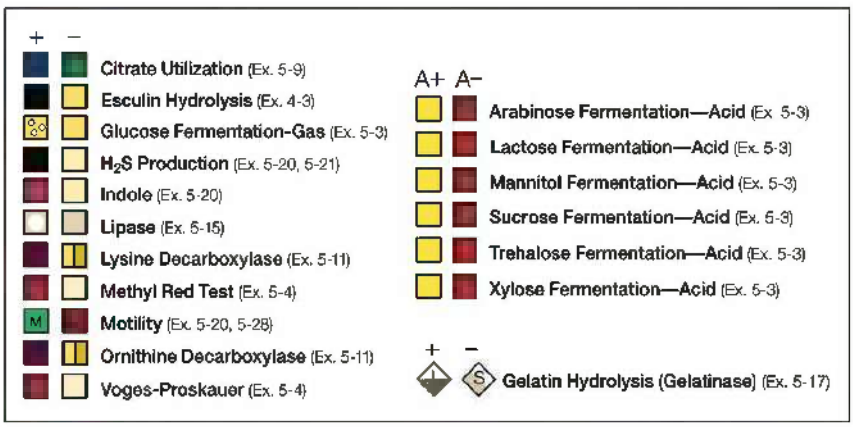
- ☐ Media listed in Table 7-6 for biochemical testing
- ☐ Pure cultures of organisms listed in Figure 7.12 to be used for positive controls and unknowns (All organisms are available from either Ward's or Carolina Biological Supply Company. BSL-2 organisms should not be chosen as unknowns by schools or classes not adequately prepared to handle them.)

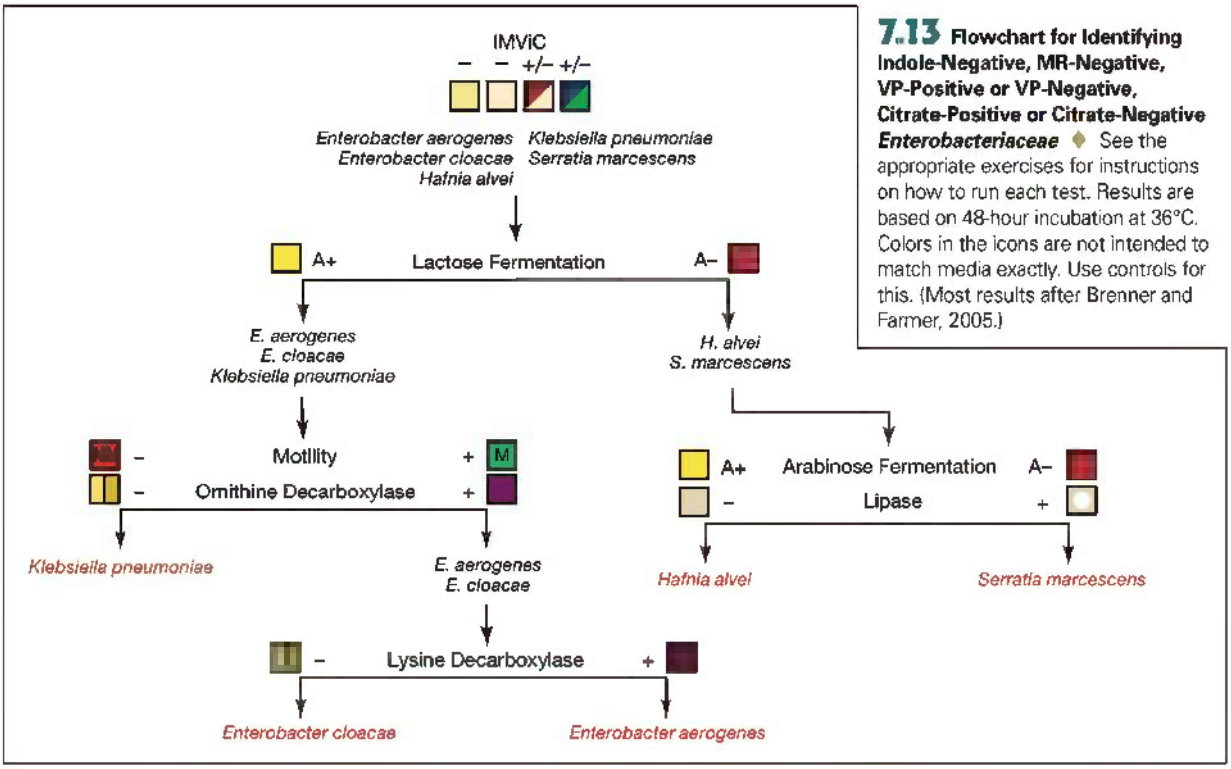
Per Student

- ☐ Two MacConkey agar plates
- ☐ One unknown organism[1] from the list in Figure 7.12
- ☐ TSA slants (enough to keep the culture fresh over the time required for identification)
- ☐ Gram-stain kit
- ☐ Microscope slides
- ☐ Compound microscope with oil-objective lens and ocular micrometer
- ☐ Immersion oil
- ☐ Lens paper
- ☐ Gloves
- ☐ Sterile wooden sticks or disposable loops

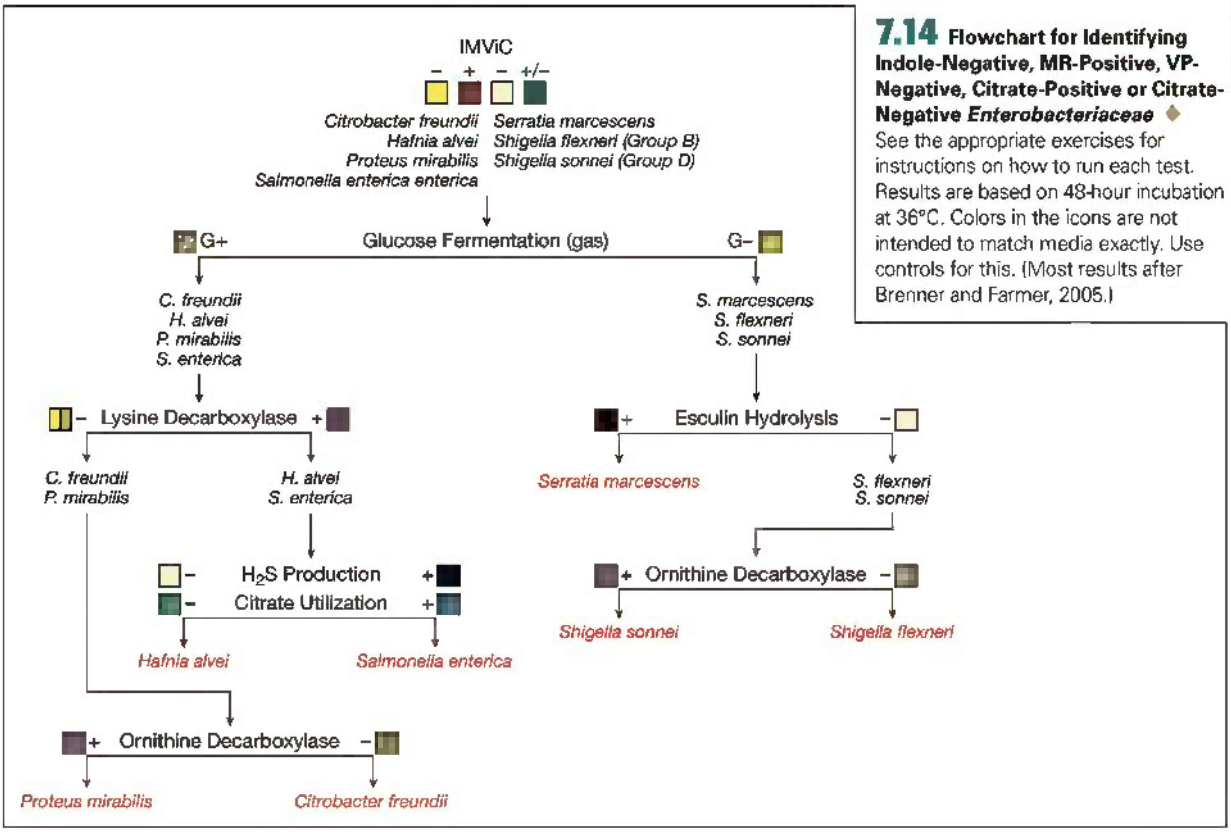
[1] ***Note to instructor:*** This exercise can be combined with Exercises 7-8 or 7-9 by passing out a mixture of an enteric and a Gram-positive coccus or rod as unknowns. The student must isolate the two organisms from mixed culture and then proceed to identify the two independently. If this option is chosen, grow the two unknowns separately and then mix them immediately prior to handing them out in lab.

TABLE **7-6** **Key to Icons Used in This Exercise** ♦ Colors are to remind you what results look like. They should not be used to compare with your results. ***Note:*** For the fermentation tests, "A+" means acid is produced; "A−" means no acid is produced.

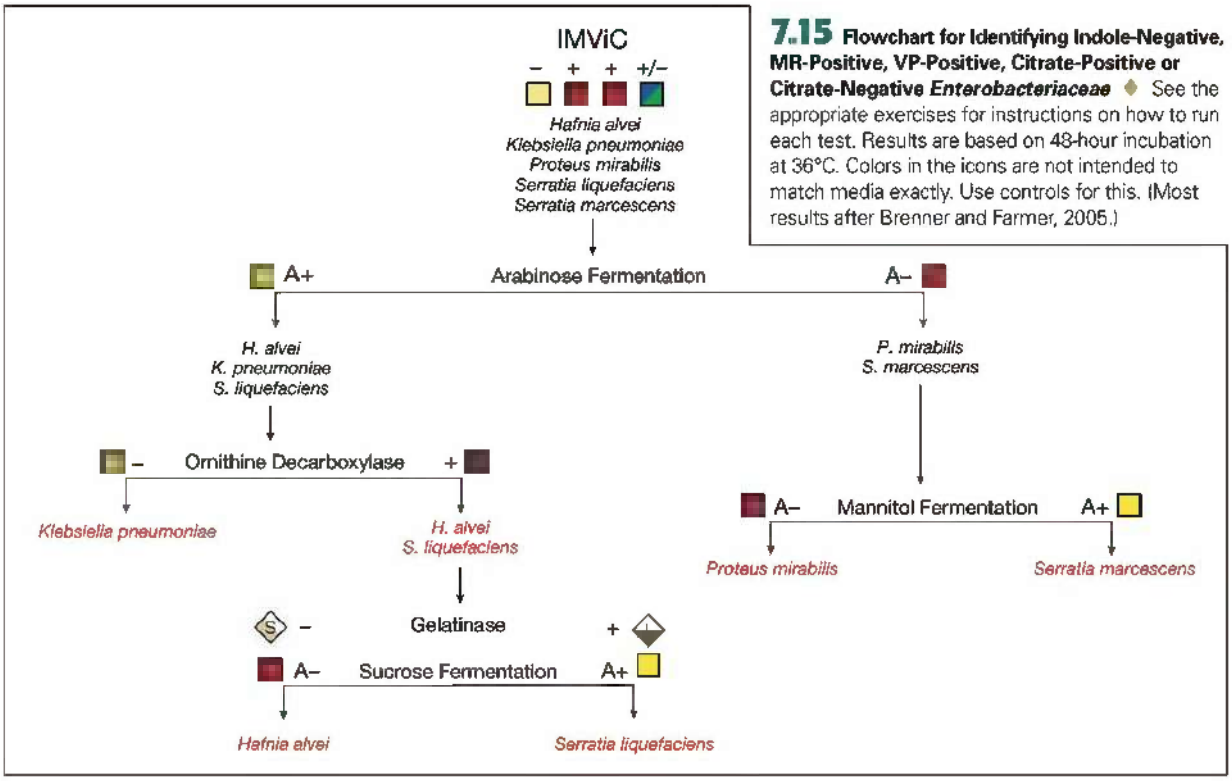




7.13 Flowchart for Identifying Indole-Negative, MR-Negative, VP-Positive or VP-Negative, Citrate-Positive or Citrate-Negative *Enterobacteriaceae* ♦ See the appropriate exercises for instructions on how to run each test. Results are based on 48-hour incubation at 36°C. Colors in the icons are not intended to match media exactly. Use controls for this. (Most results after Brenner and Farmer, 2005.)



7.14 Flowchart for Identifying Indole-Negative, MR-Positive, VP-Negative, Citrate-Positive or Citrate-Negative *Enterobacteriaceae* ♦ See the appropriate exercises for instructions on how to run each test. Results are based on 48-hour incubation at 36°C. Colors in the icons are not intended to match media exactly. Use controls for this. (Most results after Brenner and Farmer, 2005.)

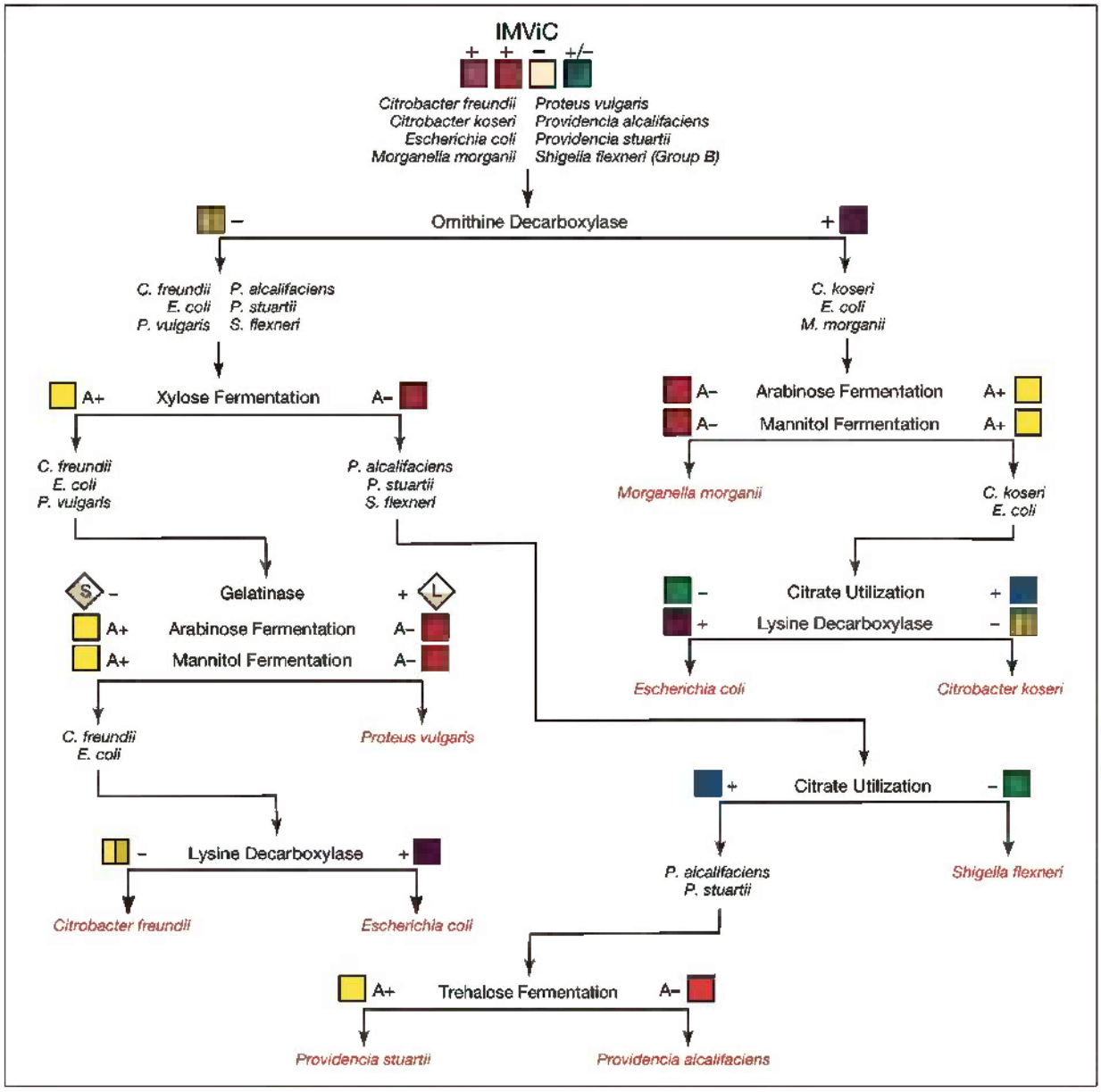


7.15 Flowchart for Identifying Indole-Negative, MR-Positive, VP-Positive, Citrate-Positive or Citrate-Negative *Enterobacteriaceae* ♦ See the appropriate exercises for instructions on how to run each test. Results are based on 48-hour incubation at 36°C. Colors in the icons are not intended to match media exactly. Use controls for this. (Most results after Brenner and Farmer, 2005.)

7

Procedure

1. Obtain an unknown. Record its number on the data sheet, page 563.
2. **All procedures should be run using BSL-2 precautions unless otherwise directed by your instructor.**
3. Streak the sample for isolation on two MacConkey agar plates. Incubate one at 25°C and the other 35 ± 2°C for 24 hours or more.
4. It is best to check your plate for isolation at 24 hours. If you have isolation, continue with step 5. If you do not have time to continue with step 5, refrigerate your plates until you do. If you do not see isolation, ask your instructor if you should re-streak for isolation, let the original plates continue to incubate, or do both. Record your isolation procedure as directed on the data sheet.
5. Examine the plates and record optimum growth temperature under Preliminary Observations on the data sheet. Find a well-isolated colony on one of the plates and record its morphology (including medium and incubation temperature) on the data sheet. Also include the result of the differential component of MacConkey agar as Test #1 under Differential Tests on the back of the data sheet. Continue to record your isolation procedure on the data sheet.
6. Perform a Gram stain on a portion of one well-isolated colony. Record its Gram reaction and cell morphology, arrangement, and size on the data sheet under Preliminary Observations. If the isolate is a Gram-negative rod, transfer a portion of the *same* colony to a tryptic soy agar slant (or another suitable growth medium as available in your lab) and incubate it at its optimum temperature. This is your pure culture to be used as a source of organisms for further testing. Complete the record of your isolation procedure on the data sheet.
7. Perform an oxidase test on what remains of the isolated colony, or wait until your pure culture has grown and do it then. Record the result as Test #2 under Differential Tests on the data sheet. If the isolate grew on MacConkey agar and is an oxidase-negative, Gram-negative rod, it probably is a member of the *Enterobacteriaceae*. At the discretion of your instructor, further tests chosen from the characteristics listed under Theory may be run to assure that it is an organism from *Enterobacteriaceae*.



7.16 Flowchart for Identifying Indole-Positive, MR-Positive, VP-Negative, Citrate-Positive or -Negative *Enterobacteriaceae* ◆
See the appropriate exercises for instructions on how to run each test. Results are based on 48-hour incubation at 36°C. Colors in the icons are not intended to match media exactly. Use controls for this. (Most results after Brenner and Farmer, 2005.)

8 You now will begin biochemical testing to identify your organism. The tests in the flowcharts were chosen because of their uniformity of results as published in standard microbiological references, so they give the best chance of correct identification. Be aware, however, that the symbol "+" indicates that 90% or more of the strains tested give a positive result. This means that as many as 10% of the strains tested give a negative result. The same is true of the symbol "−"; that is, as many as 10% of the strains give a positive result for that test. Bottom line: There are no guarantees that your particular lab strains will behave in the majority, and if not, you will misidentify your unknown. This issue, if relevant to your unknown, will be addressed in step 12.

9 Perform the IMViC series of tests (Indole Production, Methyl Red Test, Voges-Proskauer Test, and Citrate Utilization) at your isolate's optimum temperature. You may run these simultaneously.
   - Use Test #3 for indole, Test #4 for MRVP, and Test #5 for citrate.
   - Record the relevant inoculation information and results on the data sheet under Differential Tests.
   - Match your isolate's IMViC results with one shown in Figure 7.12, and proceed to the appropriate identification flowchart (Figs. 7.13 through 7.16). Record the figure number of the flowchart you will use on the data sheet. (*Note*: If a result is shown as +/− [as it is for all citrate tests], either result for that test is a match.)

10 Follow the tests in the appropriate flowchart to identify your unknown. Use these guidelines.
   - Do not run more than one test at a time (except for the IMViC) unless your instructor tells you to do so.
   - Where multiple tests are listed at a branch point, run only one, and then move to the next level in the flowchart as indicated by the result of that test.
   - Continue to record the relevant inoculation information and results on the data sheet as you go. Keep accurate records of what you have done. Do not enter all your data at the end of the project.

11 When you have identified your organism, use *Bergey's Manual* or another standard reference to find one more test to run for confirmation. The confirmatory test does not have to separate the final organisms on the flowchart. It only has to be a test for which you know the result and that you have not run already as part of the identification process. Record this test on the data sheet and identify it as the confirmatory test. Record the *expected* result on the Comments line. After incubation, note whether the organism's result matches or differs from the expected result.

12 After the confirmatory test, write the name of the organism on the back of the data sheet under "My unknown is" and check with your instructor to see if you are correct. If you are, congratulations! If you are not, your instructor will advise you as to which test(s) gave "incorrect" results by writing the test name(s) in the "rerun" space next to your identification. These should be rerun with appropriate controls from your school's inventory of organisms. Record the test(s) and control(s) on your data sheet. Checking the results with controls and your unknown will indicate if the "incorrect" test result was truly incorrect when run previously or if the strain of organism your school is using doesn't match the majority of strains for that test result.

References

Brenner, Don J. and J. J. Farmer, III. Order XIII. "Enterobacteriales" in *Bergey's Manual of Systematic Bacteriology*, Vol. 2, *The Proteobacteria, Part B—The Gammaproteobacteria*. New York: Springer, 2005.

Farmer III, J. J., K. D. Boatwright, and J. Michael Janda. Chap. 42 in *Manual of Clinical Microbiology*, 9th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Marie Louise Landry, and Michael A. Pfaller, eds. Washington, DC: ASM Press, 2007.

Forbes, Betty A., Daniel F. Sahm, and Alice S. Weissfield. Chap. 25 in *Bailey & Scott's Diagnostic Microbiology*, 11th ed. St. Louis, MO: Mosby, 2002.

MacFaddin, Jean F. *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

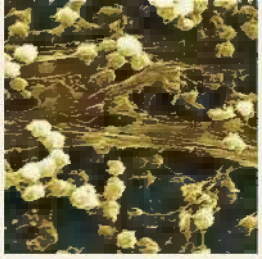
Winn Jr., Washington, Stephen Allen, William Janda, Elmer Koneman, Gary Procop, Paul Schreckberger, and Gail Woods. Chap. 6 in *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. Philadelphia: Lippincott Williams & Wilkins, 2006.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-7

Identification of *Enterobacteriaceae*

ISOLATION PROCEDURE

Unknown Number _____

Record all activities associated with isolation of your organisms—from mixed culture to pure culture. Always include the date, source of inoculum, destination, type of inoculation, incubation temperature, and any other relevant information. Also make note of transfers made to keep your pure culture fresh. (This log must be kept current.)

Preliminary Observations

Colony Morphology (include medium and incubation temperature) _____

Gram Stain _____ Cell Dimensions _____ Optimum Temperature _____

Cellular Morphology and Arrangement _____

Differential Tests

Begin recording with your MacConkey result, followed by the Oxidase test, and then your IMViC results. This log must be kept current.

Record the Identification Chart you are using. If you change charts, indicate the figure number of the new chart and the date of the change.

Test #1: Date Begun: Date Read: Result:

Comments:

Test #2: Date Begun: Date Read: Result:

Comments:

Test #3: Date Begun: Date Read: Result:

Comments:

Test #4: Date Begun: Date Read: Result:

Comments:

Test #5: Date Begun: Date Read: Result:

Comments:

Test #6: Date Begun: Date Read: Result:

Comments:

Test #7: Date Begun: Date Read: Result:

Comments:

Test #8: Date Begun: Date Read: Result:

Comments:

Test #9: Date Begun: Date Read: Result:

Comments:

Test #10: Date Begun: Date Read: Result:

Comments:

Test #11: Date Begun: Date Read: Result:

Comments:

Test #12: Date Begun: Date Read: Result:

Comments:

My Unknown is: Rerun[1]:

Rerun:

Rerun:

[1] Your instructor will write what tests to rerun in this space if you misidentify your unknown.

Identification of Gram-positive Cocci

EXERCISE 7-8

Theory

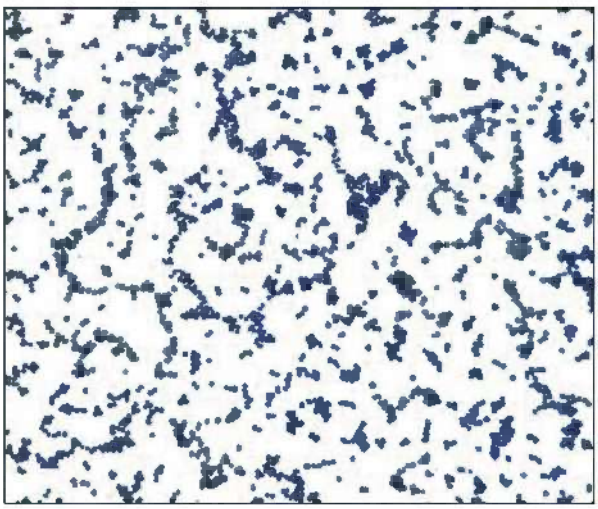
Gram-positive cocci are frequent isolates in a clinical setting because they are common inhabitants of skin and mucous membranes. Five genera, representing very diverse groups, are briefly described below and will be used in this lab exercise.

Staphylococcus (Fig. 7.17)

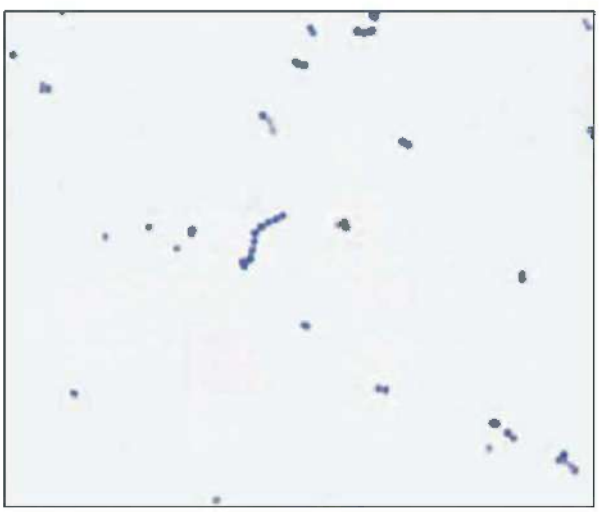
- Phylum *Firmicutes*, Order *Bacillales*
- Gram-positive cocci in singles, pairs, tetrads, or clusters (especially when grown in broth)
- Catalase positive
- With rare exception, facultatively anaerobic
- Most are oxidase negative
- G + C content within the genus ranges between 27% and 41%.
- Grow in 6.5% NaCl
- Most produce acid from glucose
- Most are resistant to bacitracin
- Key pathogen is *Staphylococcus aureus* (toxic shock syndrome and a variety of other skin and deep organ infections, including bacteremia)

Streptococcus (Fig. 7.18)

- Phylum *Firmicutes*, Order *Lactobacillales*
- Gram-positive cocci to ovoid cocci in singles, pairs, or short chains (especially when grown in broth)
- Gray to white, moist colonies are frequently observed
- Catalase negative
- Oxidase negative
- Facultatively anaerobic
- Nutritionally fastidious
- Some require 5% CO_2 for growth
- Ferment glucose and other carbohydrates, mostly to lactic acid
- G + C content within the genus ranges between 33% and 46%
- Many species produce hemolysins that either completely (β-hemolysins) or partially (α-hemolysins) destroy erythrocytes; some species are nonhemolytic
- Beta-hemolytic streptococci have traditionally been grouped by antigens first described by Rebecca Lancefield; important Lancefield groups include Group A (*S. pyogenes*) and Group B (*S. agalactiae*)
- Key pathogens are *Streptococcus pyogenes* (strep throat, necrotizing fasciitis, scarlet fever) and *S. pneumoniae* (bacterial pneumonia, otitis media, and bacteremia)



7.17 *Staphylococcus aureus* ◆ This specimen grown in broth illustrates the grapelike clusters of cells characteristic of the genus. Specimens grown on solid media may not show the clusters as clearly.



7.18 *Streptococcus agalactiae* ◆ This specimen grown in broth illustrates the streptococcal arrangement of cells characteristic of the genus. Specimens grown on solid media may not show the chains as clearly.

Enterococcus

- Phylum *Firmicutes*, Order *Lactobacillales*
- Formerly members of the genus *Streptococcus*
- Gram-positive cocci to ovoid cocci in singles, pairs, or short chains (especially when grown in broth); may be more rod-shaped if grown on solid media
- Catalase negative
- Oxidase negative
- Facultatively anaerobic
- Lactic acid, but no gas, is the sole end product of fermentation
- Grow in 6.5% NaCl broth
- Grow in bile esculin
- Most are PYR positive
- G + C content within the genus ranges between 35% and 45%
- Most express the Lancefield Group D antigen
- Most species are commensals or opportunistic pathogens; the key opportunistic pathogen is *E. faecalis* (urinary tract infections, wound infections, and bacteremia in seriously ill elderly persons)

Micrococcus

- Phylum *Actinobacteria*, Order *Micrococcales*
- Gram-positive cocci in pairs and tetrads
- Obligately aerobic
- Oxidase positive
- Do not produce acid from glucose
- Bacitracin susceptible
- Catalase positive
- G + C content within the genus ranges between 69% and 76%.
- Grow in 6.5% NaCl
- Commensals or opportunistic pathogens. *M. luteus* is a common skin commensal

Kocuria

- Phylum *Actinobacteria*, Order *Micrococcales*
- A small genus; most species were formerly classified in the genus *Micrococcus*
- Gram-positive cocci in pairs and tetrads
- Obligately aerobic
- Do not produce acid from glucose
- Bacitracin susceptible
- Catalase positive
- G + C content within the genus ranges between 66% and 75%.
- Commensals or opportunistic pathogens, especially among immunocompromised patients

The organisms to be used as unknowns are listed in Table 7-7, as are the tests to be used in their identification. (***Note:*** Your instructor will choose Gram-positive coccus unknowns appropriate to your microbiology course and facilities.)

Application

Identification of Gram-positive cocci from human specimens requires a coordinated and integrated use of biochemical tests and stains. Although several serological tests allow rapid identification, flowcharts are still a useful way to visualize the process of identification by elimination.

TABLE 7-7 List of Organisms and Key to Icons Used in This Exercise ♦ The organisms listed are Gram-positive cocci available from Ward's or Carolina Biological Supply companies. Icon colors are to remind you what the results look like. They should not be used to compare with your results. ***Note:*** For the fermentation tests, "A+" means acid is produced; "A−" means no acid is produced.

Gram-Positive Cocci and Identification Tests

Catalase-Positive (Figure 7.19)	Catalase-Negative (Figure 7.20)
Kocuria rosea (= *Micrococcus roseus*)	*Enterococcus faecalis* (BSL-2)
Micrococcus luteus	*Streptococcus agalactiae* (BSL-2)
Staphylococcus aureus (BSL-2)	*Streptococcus dysgalactiae* subsp. *equisimilis* (BSL-2)
Staphylococcus epidermidis (BSL-2)	(= *Streptococcus equisimilis*)
Staphylococcus saprophyticus	*Streptococcus mutans*
	Streptrococcus salivarius
	Streptococcus sanguinis (BSL-2)
	Streptococcus pneumoniae (BSL-2)
	Streptococcus pyogenes (BSL-2)

+ −
Arginine Decarboxylase (Dihydrolase) (Ex. 5-11)
CAMP Test (Ex. 5-26)
Catalase (Ex. 5-6)
NO_3 reduced to NO_2 (Ex. 5-8)
PYR Test (Ex. 5-19)
Starch Hydrolysis (Amylase) (Ex. 5-13)
Voges-Proskauer (Ex. 5-4)

β α γ
Hemolysis (Ex. 5-25)

A+ A−
Mannitol Fermentation—Acid (Ex. 5-3)

O F
Glucose O-F Test (Ex. 5-2)

S R
≥10 mm / ≤9 mm Bacitracin Susceptibility (0.04 U) (Ex. 5-24)
>16 mm / ≤16 mm Novobiocin Susceptibility (0.5 µg) (Ex. 5-24)
>14 mm / <14 mm Optochin Susceptibility (5 µg) (Ex. 5-24)

+ −
Coagulase (Ex. 5-27)
Gelatin Hydrolysis (Gelatinase) (Ex. 5-17)

In This Exercise

This exercise will span several lab periods. You will be given an unknown Gram-positive coccus from the organisms listed in Table 7-7, and then run biochemical tests as directed by the flowcharts in Figure 7.19 or Figure 7.20 to identify it.

✓ Materials

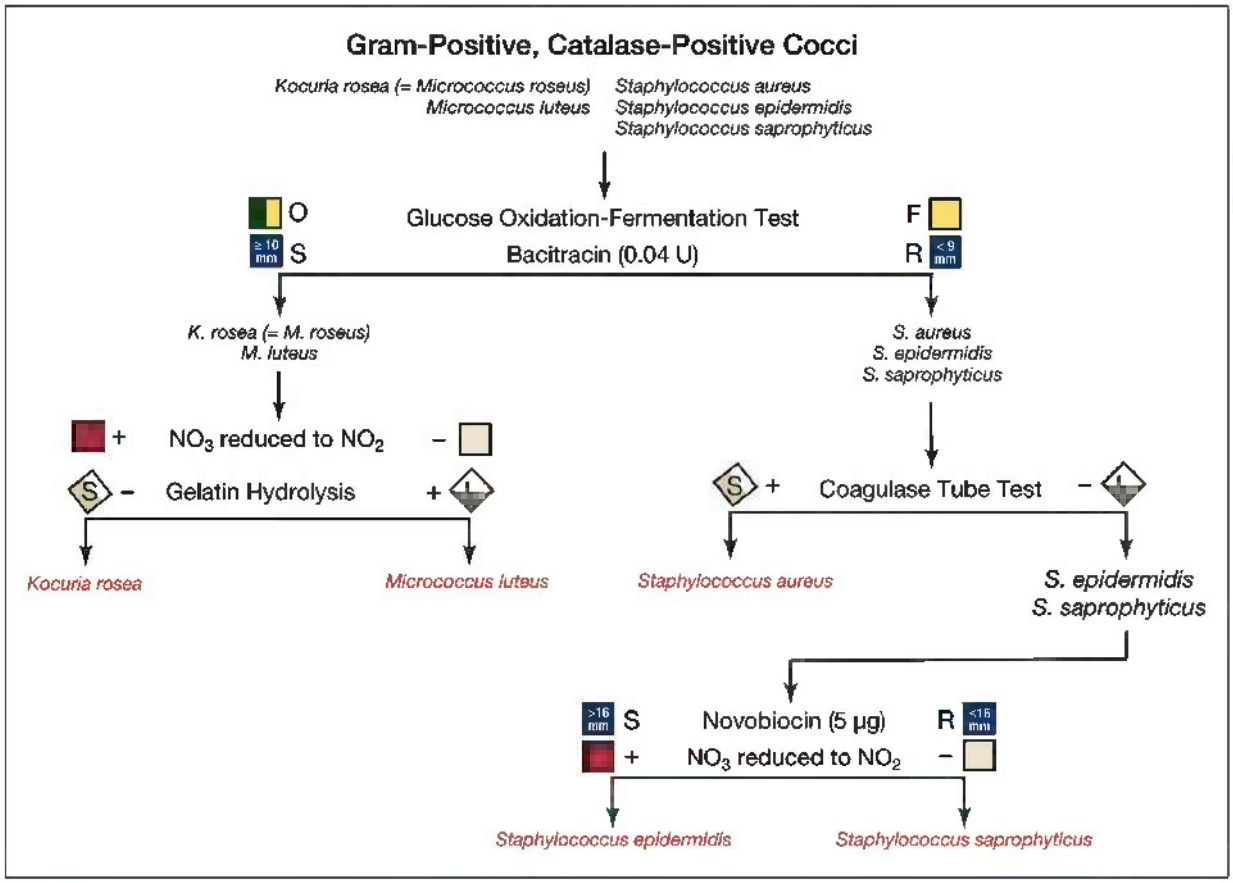
Per Class

- ☐ Media listed in Table 7-7 for biochemical testing
- ☐ Pure cultures of organisms listed in Table 7-7 to be used for positive controls and unknowns (BSL–2 organisms should not be chosen by schools or classes not adequately prepared to handle them.)
- ☐ Todd-Hewitt broth or brain–heart infusion broth
- ☐ (Optional) candle jar setup
- ☐ (Optional) CO_2 incubator

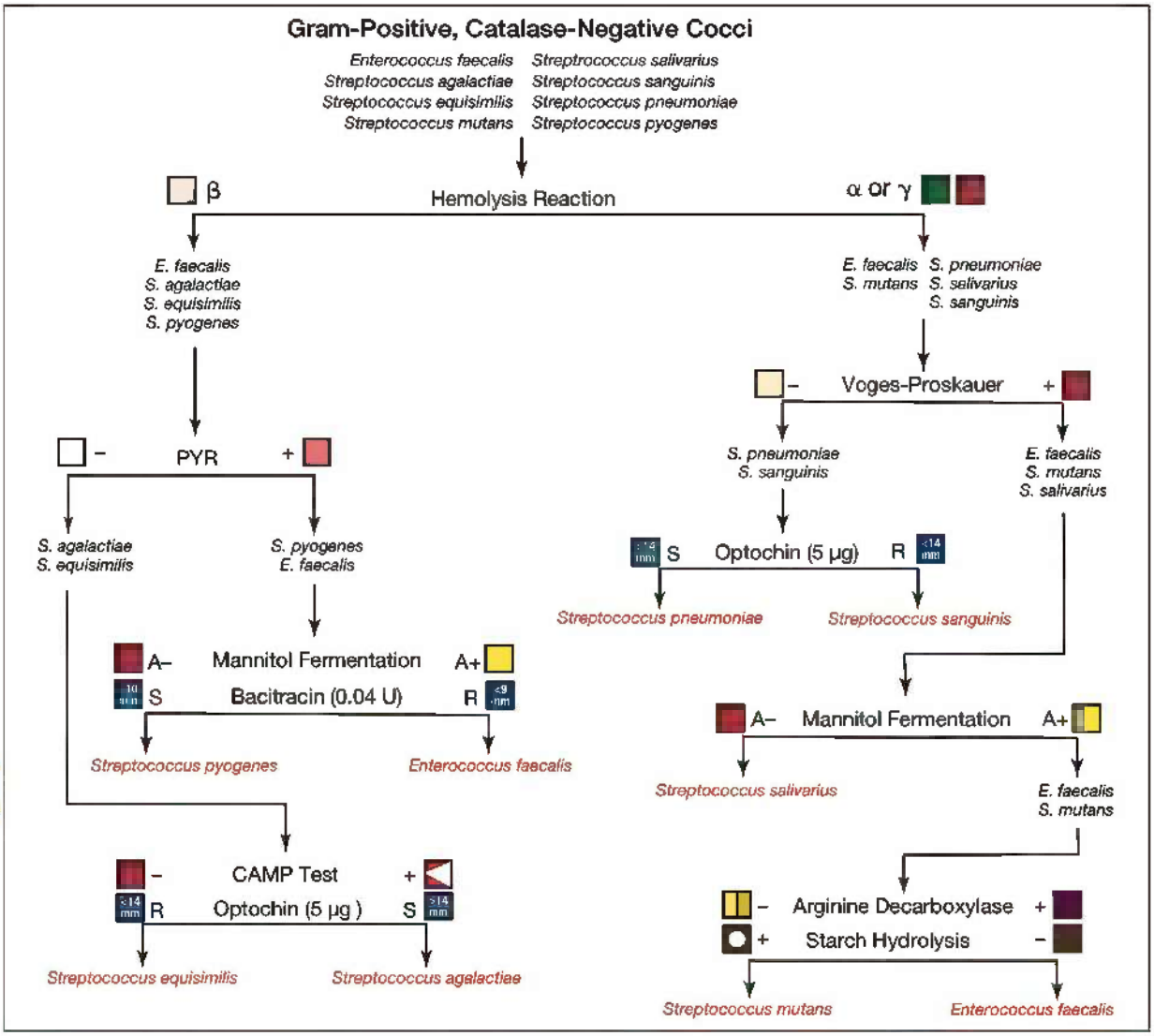
Per Student

- ☐ Two PEA, CNA, or 5% sheep blood agar plates
- ☐ Gram-stain kit
- ☐ Sterile wooden sticks or disposable loops
- ☐ TSA slants (enough to keep the culture fresh over the time required for identification)
- ☐ Gloves
- ☐ Microscope slides
- ☐ 3% H_2O_2
- ☐ One unknown organism[1] from the list in Table 7-7
- ☐ Compound microscope with oil-objective lens and ocular micrometer
- ☐ Lens paper
- ☐ Immersion oil

[1] ***Note to instructor:*** This exercise can be combined with Exercise 7-7 by passing out a mixture of a Gram-negative enteric and a Gram-positive coccus as unknowns. The student then must isolate the two organisms from a mixed culture, and then proceed to identify the two independently. If this option is chosen, grow the two unknowns separately, and then mix them immediately prior to handing them out in lab.



7.19 Identification Flowchart for Gram-positive, Catalase-Positive Cocci (Usually in Tetrads or Clusters) ◆ See the appropriate exercises for instructions on how to run each test. In some cases, final identification to species is difficult. Try to get that far, but you may reach a point where your lab strains cannot be differentiated any further. (Compiled from sources listed in the References.)



7.20 Identification Flowchart for Gram-positive, Catalase-Negative Cocci (Usually in Pairs or Chains) ◆ See the appropriate exercises for instructions on how to run each test. In some cases, final identification to species is difficult. Try to get that far, but you may reach a point where your lab strains cannot be differentiated any further.

(Compiled from sources listed in the References.

7

Procedure

1. Obtain an unknown. Record its number on the data sheet, page 571.
2. **All procedures should be run using BSL-2 precautions unless otherwise directed by your instructor.**
3. Streak the sample for isolation on two sheep blood agar, PEA, or CNA plates. Incubate one in the candle jar and the other aerobically. Incubate both at 30°C–35°C.
4. It is best to check your plate for isolation at 24 hours. If you have isolation, continue with step 5. If you do not have time to continue with step 5, refrigerate your plates until you do. If you do not see isolation, ask your instructor if you should re-streak for isolation, let the original plates continue to incubate, or do both. Record your isolation procedure as directed on the data sheet.

5 Examine the plates and record optimum growth temperature and CO_2 requirement under Preliminary Results on the data sheet. Find a well-isolated colony on one of the plates and record its morphology (including medium and incubation temperature) on the data sheet. Also record the result of the differential component of blood agar as Test #1 on the back of the data sheet if you used sheep blood agar. Continue to record your isolation procedure on the data sheet.

6 Perform a Gram stain on a portion of one well-isolated colony. Continue testing colonies until you find one that is a Gram-positive coccus.

- Record its Gram reaction and cell morphology, arrangement and size under Preliminary Observations on the data sheet.
- If the isolate is a Gram-positive coccus, transfer a portion of the *same* colony to a Todd-Hewitt broth (or another suitable growth medium as available in your lab) and incubate it at 35 ± 2°C. This is your pure culture to be used as a source of organisms for further testing. Complete the record of your isolation procedure on the data sheet.

7 Perform a catalase test on what remains of the isolated colony, or wait until your pure culture has grown and do it then. Record the result as Test #1 (or #2 if hemolysis reaction is already recorded) under Differential Tests on the data sheet. (*Note*: Be sure to use growth from the top of the colony if isolation was performed on a blood agar plate. This minimizes the possibility of a false positive from the catalase-positive erythrocytes in the medium. Alternatively, you can postpone the catalase test until your pure culture has grown.)

8 Use the chart in Figure 7.19 for identification of catalase-positive cocci (usually in tetrads or clusters). Use the chart in Figure 7.20 if the isolate is catalase-negative (usually with cocci in pairs or chains). Record the figure number of the flowchart you will use on the data sheet. If your isolate is catalase-negative, ask your instructor if you would get better growth of your pure culture using Todd-Hewitt broth or brain–heart infusion broth and incubating in a candle jar or CO_2 incubator.

9 Now you will begin biochemical testing to identify your organism. The tests in the flowcharts were chosen because of their uniformity of results as published in standard microbiological references, so they give the best chance for correct identification. Be aware, however, that the symbol "+" indicates that 90% or more of the strains tested give a positive result. This means that as many as 10% of the strains tested give a negative result. The same is true of the symbol "−". That is, as many as 10% of the strains give a positive result. Bottom line: There are no guarantees that your lab strains will behave in the majority, and if not, you will misidentify your unknown. This issue, if relevant to your unknown, will be addressed in step 12.

10 Follow the tests in the appropriate flowchart to identify your unknown.[2] Use these guidelines.

- Do not run more than one test at a time unless your instructor tells you to do so.
- Where multiple tests are listed at a branch point, run only one, and then move to the next level in the flowchart as indicated by the result of that test.
- Continue to record the relevant inoculation information and results on the data sheet as you go. Keep accurate records of what you have done. *Do not* enter all your data at the end of the project.

11 When you have identified your organism, use *Bergey's Manual* or another standard reference to find one more test to run for confirmation. The confirmatory test does not have to separate the final organisms on the flowchart. It only has to be a test for which you know the result and that you have not run already as part of the identification process. Record this test on the data sheet, and identify it as the confirmatory test. Record the *expected* result on the comments line. After incubation, note whether the organism's result matches or differs from the expected result.

12 After the confirmatory test, write the organism's name on the back of the data sheet under "My Unknown is" and check with your instructor to see if you are correct. If you are, congratulations! If you are not, your instructor will advise you as to which test(s) gave "incorrect" results by writing the test name(s) in the "rerun" space next to your identification. These should be rerun with appropriate controls from your school's inventory of organisms. Record the test(s) and control(s) on your data sheet. Checking the results with controls and your unknown will indicate if the "incorrect" test result was truly incorrect when previously run, or if the strain of organism your school is using does not match the majority of strains for that test result.

[2] These flowcharts can be used only for the organisms listed in Table 7-7.

References

Becker, Karsten and Christof von Eiff. Chap. 19 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

MacFaddin, Jean F. *Biochemical Tests for Identification of Medical Bacteria,* 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Ruoff, Kathryn L. Chap. 22 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Spellerberg, Barbara and Claudia Brandt. Chap. 20 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Švec, Pavel and Luc A. Devriese. Pages 594–607 in *Bergey's Manual of Systematic Bacteriology,* 2nd ed. Vol. 3, The Firmicutes. New York: Springer, 2009.

Teixeira, Lúcia Martins, Maria S Glória Siqueira Carvalho, Patricia Lynn Shewmaker, and Richard R. Facklam. Chap. 21 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Teuber, Michael. Pages 711–722 in *Bergey's Manual of Systematic Bacteriology,* 2nd ed., Vol. 3, The Firmicutes. New York: Springer, 2009.

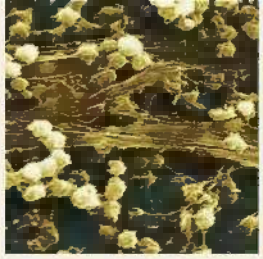
Whiley, Robert A. and Jeremy M. Hardie. Pages 655–711 in *Bergey's Manual of Systematic Bacteriology,* 2nd ed., Vol. 3, The Firmicutes. New York: Springer, 2009.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-8

Identification of Gram-positive Cocci

ISOLATION PROCEDURE

Unknown Number _____

Record all activities associated with isolation of your organisms—from mixed culture to pure culture. Always include the date, source of inoculum, destination, type of inoculation, incubation temperature, and any other relevant information. Also make note of transfers made to keep your pure culture fresh. (This log must be kept current.)

Preliminary Observations

Colony Morphology (include medium and incubation temperature) _____

Gram stain _____ Cell dimensions _____ CO_2 requirement _____

Cell morphology and arrangement _____ Optimum temperature _____

Differential Tests

Begin recording with your Catalase test (and hemolysis result, if determined). This log must be kept current.

Record the Identification Chart you are using. If you change charts, indicate the figure number of the new chart and the date of the change.

Test #1: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #2: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #3: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #4: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #5: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #6: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

7 **Test #7:** _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #8: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #9: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #10: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #11: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

Test #12: _____ Date Begun: _____ Date Read: _____ Result: _____

Comments: _____

My Unknown is: _____ Rerun[1]: _____

_____ Rerun: _____

_____ Rerun: _____

[1] Your instructor will write what tests to rerun in this space if you misidentify your unknown.

Identification of Gram-positive Rods

EXERCISE 7-9

Theory

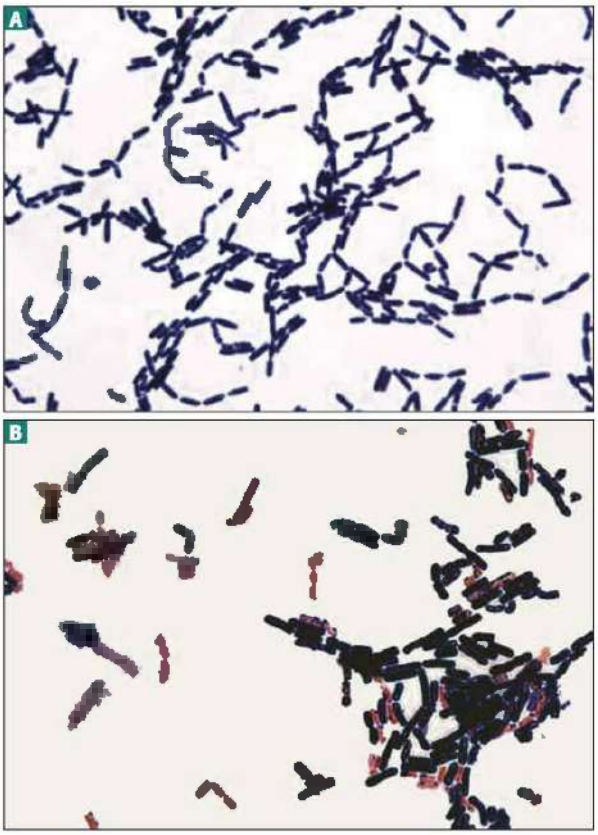
Several genera of Gram-positive rods have environmental, industrial, and clinical importance. Among these are *Bacillus*, *Clostridium*, *Corynebacterium*, *Lactobacillus*, and *Mycobacterium*. Each is briefly characterized below.

Bacillus (Fig. 7.21)

- Phylum *Firmicutes*, Order *Bacillales*
- A large, heterogeneous group comprising 162 species (as of 2011)
- Gram-positive rods (at least early in growth—Fig. 7.21A), in singles or chains
- Aerobic or facultatively anaerobic
- Produce endospores aerobically; spore shape and position are variable, but never more than one per cell
- Most are catalase positive
- Most are motile
- Most are soil saprophytes and are chemoorganotrophic
- Key pathogens are *B. anthracis* (anthrax) and *B. cereus* (food poisoning and opportunistic infections)

Lactobacillus (Fig. 7.22)

- Phylum *Firmicutes*, Order *Lactobacillales*
- Gram-positive rods, sometimes in chains
- Faculatively anaerobic with fermentative metabolism; lactic acid is an abundant product
- Catalase negative
- Oxidase negative
- No endospores
- Nonmotile
- Isolated from a variety of foods (dairy, fish, grain, meat), and many are in normal flora (mouth, intestines, and vagina)
- No key pathogens and therefore not usually identified to species in clinical setting



7.21 Gram Stains of *Bacillus cereus* ◆ (A) This Gram stain of a young (<24 hours old) *B. cereus* culture stains uniformly Gram-positive. (B) With age, many *Bacillus* species lose their ability to retain the crystal violet during a Gram stain and appear pink, some with purple spots, as in this specimen.

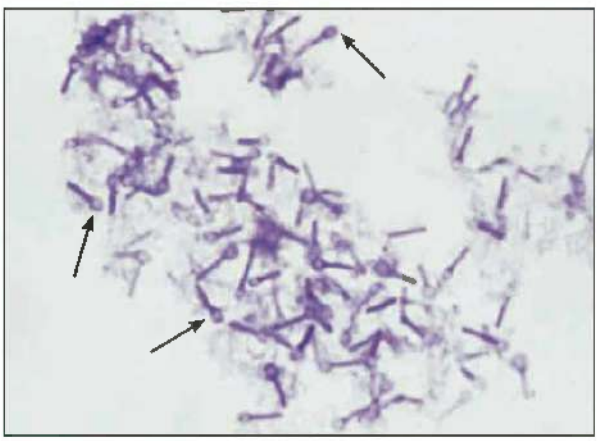
7.22 Gram Stain of *Lactobacillus* spp. ◆ Cells in the genus *Lactobacillus* range from long, slender rods to coccobacilli.

Clostridium (Fig. 7.23)

- Phylum *Firmicutes*, Order *Clostridiales*
- A large, heterogeneous genus comprising over 160 species belonging to at least 12 different groups
- Gram-positive rods (at least early in growth), in singles, pairs, or chains
- Most are obligate anaerobes, but some are microaerophiles
- Produce endospores, but not aerobically; spore shape and position are variable, but usually cause distention (bulging) of the cell
- Most are catalase negative
- Most are chemoorganotrophic and are isolated from soil, sewage, or marine sediments
- Key pathogens are *C. tetani* (tetanus), *C. botulinum* (botulism), *C. perfringens* (food poisoning and gas gangrene), and *C. difficile* (pseudomembranous colitis)

Corynebacterium (Fig. 7.24)

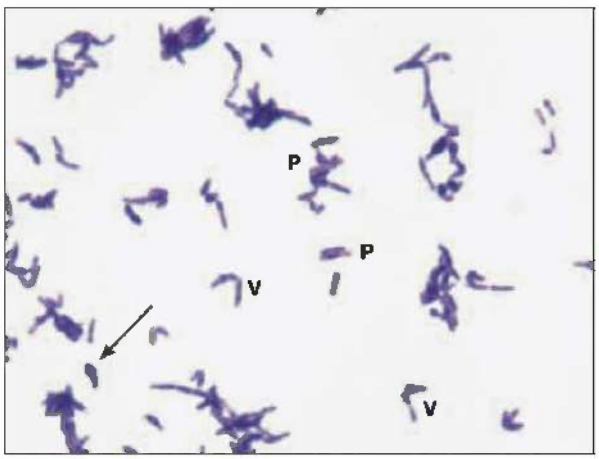
- Phylum *Actinobacteria*, Order *Corynebacteriales*
- Gram-positive rods, often club-shaped, in singles, pairs (V-forms), or arranged in stacks (palisades) or irregular clusters
- Metachromatic granules often present
- Facultatively anaerobic, but some are aerobes
- Catalase positive
- No endospores
- Nonmotile
- Some are found in the environment, and many are in the normal flora of humans (skin and mucous membranes)
- Key pathogen is *C. diphtheriae* (diphtheria), although many species are opportunistic pathogens



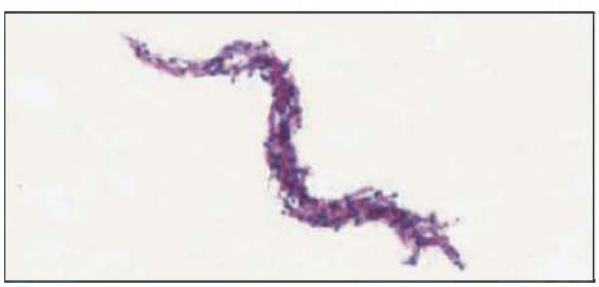
7.23 Gram Stain of *Clostridium tetani* ◆ Note the round, terminal endospores (arrows) that distend the cells.

Mycobacterium (Fig. 7.25)

- Phylum *Actinobacteria*, Order *Corynebacteriales*
- Over 130 recognized species divided into slow growers (>7 days to form colonies on Löwenstein-Jensen medium—about 70 species) and rapid growers (<7 days to form colonies on L-J medium—about 60 species)
- Weakly Gram-positive curved or straight rods, sometimes branched or filamentous
- Acid-fast (generally in early stages of growth)
- Aerobic
- Catalase positive
- Nonmotile
- No endospores
- Colony pigmentation with and without light is of taxonomic use
- Many are aquatic saprophytes
- Key pathogens are *M. tuberculosis* (tuberculosis) and *M. leprae* (leprosy or Hansen's disease). Many rapidly growing species are opportunists.

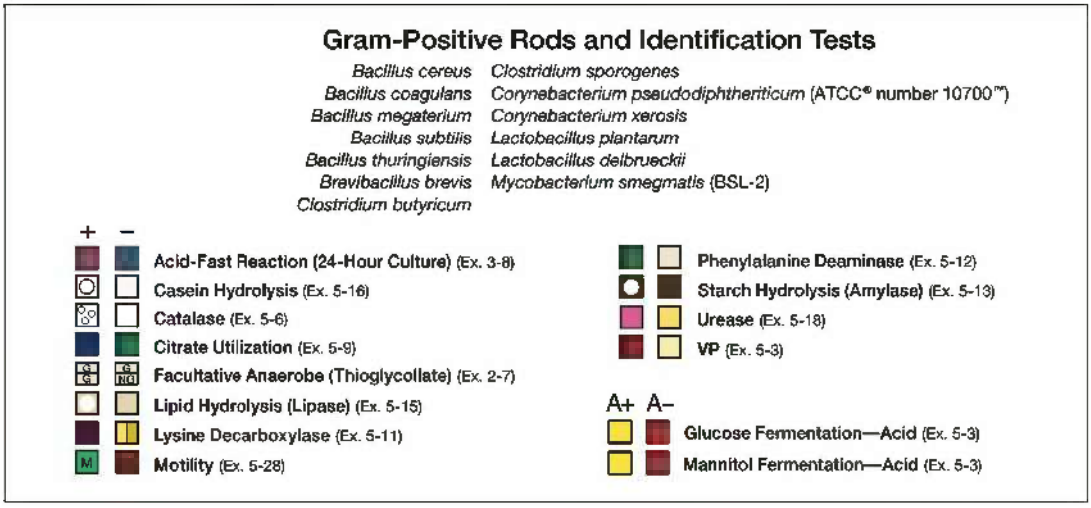


7.24 Gram Stain of *Corynebacterium diphtheria* ◆ Note the club-shaped cells that might be mistaken for spore-producers (arrow). Cells of this genus often appear as "V-shaped" pairs (V) or palisades (P).



7.25 Acid-fast Stain of *Mycobacterium tuberculosis* ◆ Note the characteristic cord-like arrangement of cells.

TABLE 7-8 List of Organisms and a Key to the Icons Used in This Exercise ◆ The organisms listed are Gram-positive rods available from Ward's or Carolina Biological Supply companies. Icon colors are to remind you what the results look like. They should not be used to compare your results. ***Note:*** For the fermentation tests, "A+" means acid is produced; "A−" means no acid is produced.



Application

Staining reactions and biochemical testing are used widely in bacterial identification.

In This Exercise

This exercise will span several lab periods. You will combine staining and biochemical testing to identify one of several unknown organisms to species level. For obvious reasons, pathogens are not included, but the organisms chosen will give you an overview of the characteristics of each genus as well as practice in the identification process.

✓ Materials

Per Class

- ☐ Media listed in Table 7-8 for biochemical testing
- ☐ Pure cultures of organisms listed in Table 7-8 to be used for positive controls and unknowns
- ☐ Anaerobic jars and GasPaks to accommodate one plate per student

Per Student

- ☐ Compound microscope with ocular micrometer and oil objective
- ☐ Immersion oil
- ☐ Clean glass slides
- ☐ Lens paper
- ☐ Gram-stain kit
- ☐ Gloves
- ☐ Sterile wooden sticks or disposable loops
- ☐ 3% H_2O_2
- ☐ One unknown organism in thioglycollate broth[1]
- ☐ Two agar plates for streaking: instructor will choose from tryptic soy agar, TSA plus 5% sheep blood, or brain–heart infusion agar
- ☐ TSA slants or thioglycollate broths (enough to keep the culture fresh over the time required for identification)

7

Procedure

1. Obtain an unknown and record its number on the data sheet, page 579.
2. Streak the sample for isolation on two agar plates (medium as provided by the instructor). Incubate both at 30°C–35°C, one aerobically and the other in the anaerobic jar. **Use BSL-2 precautions unless otherwise told by your instructor.**
3. It is best to check your plate for isolation at 24 hours. If you have isolation, continue with step 4. If you do not have time to continue with step 4, refrigerate your plates until you do. If you do not see isolation, ask your instructor if you should re-streak for isolation, let the original plates continue to incubate, or do both. Record your isolation procedure as directed on the data sheet.

[1] *Note to instructor:* This exercise can be combined with Exercises 7-7 by passing out a mixture of a Gram-positive rod and an enteric. The student must isolate the two organisms from mixed culture and then proceed to identify the two independently. If this option is chosen, grow the two unknowns separately and then mix them immediately prior to handing them out in lab.

4 After incubation examine the plates and find a well-isolated colony. Record colony morphology (including medium) under Preliminary Observations and note any differences between aerobic and anaerobic growth. Continue to record your isolation procedure on the data sheet.

5 Perform a Gram stain on a portion of one well-isolated colony.
- Record its Gram reaction and cell morphology, arrangement, and size under Preliminary Observations on the data sheet.
- If the isolate is a Gram-positive rod (it should be!), transfer a portion of the same colony to a tryptic soy agar slant (or another suitable growth medium as available in your lab) or thioglycollate broth (if anaerobic) and incubate it at 30°C–35°C. This is your pure culture to be used as a source of organisms for further testing. Complete the record of your isolation procedure on the data sheet.

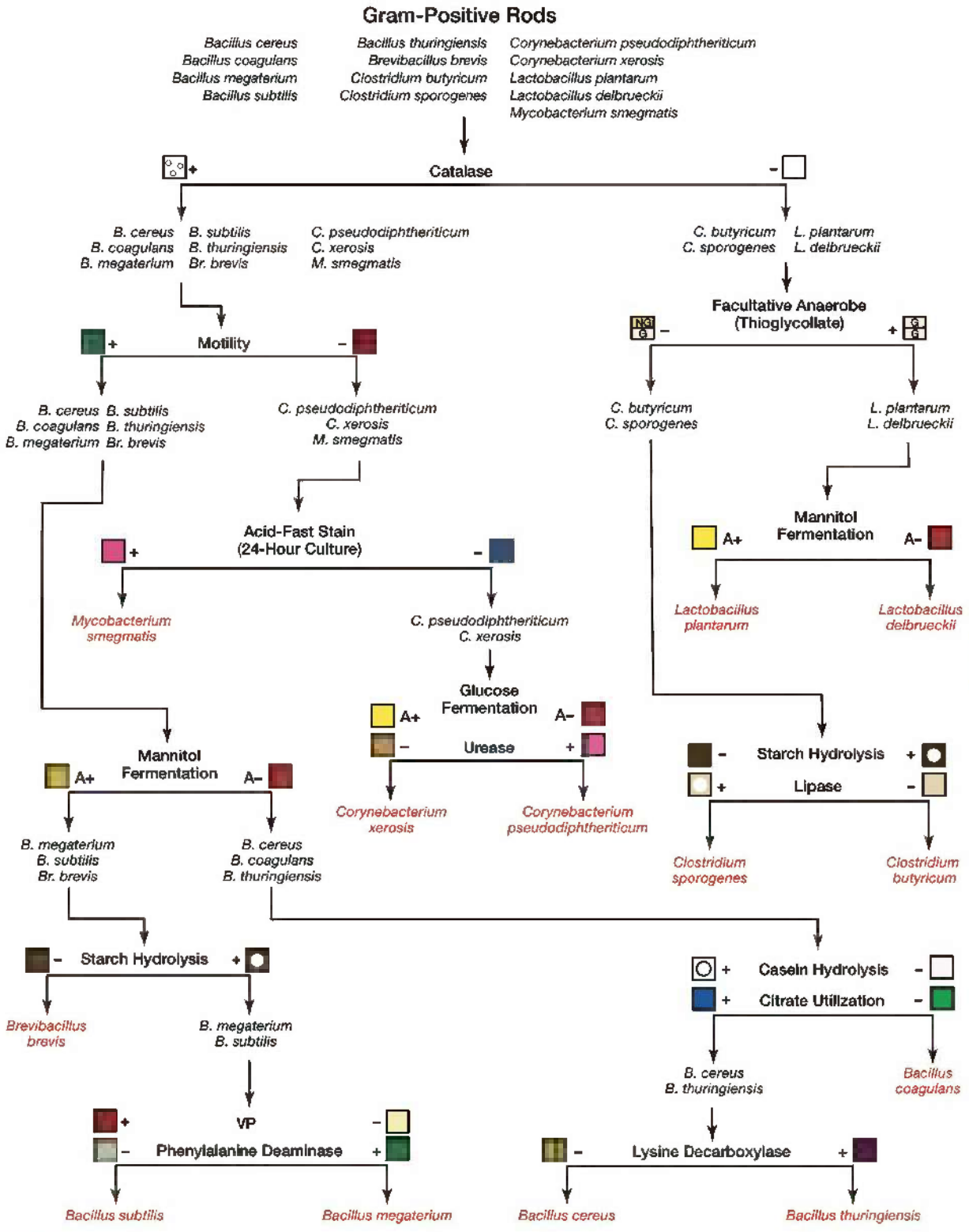
6 You now will begin biochemical testing to identify your organism. The tests in the flowchart (Fig. 7.26) were chosen because of their uniformity of results, as published in standard microbiological references, so they give the best chance of correct identification. Be aware, however, that the symbol "+" indicates that 90% or more of the strains tested give a positive result. This means that as many as 10% of the strains tested give a negative result. The same is true of the symbol "–". That is, as many as 10% of the strains give a positive result for that test. Bottom line: There are no guarantees that your particular lab strains will behave in the majority and if not, you will misidentify your unknown. This issue, if relevant to your unknown, will be addressed in step 9.

7 Follow the tests in Figure 7.26 to identify your unknown.[2] Use these guidelines.
- Do not run more than one test at a time unless your instructor tells you to do so.
- If your unknown is an obligate anaerobe, you will have to incubate media in an anaerobic jar. Coordinate your incubation with others who need the anaerobic jar to conserve GasPaks and incubator space.
- Where multiple tests are listed at a branch point, run only one, and then move to the next level in the flowchart as indicated by the result of that test.
- Continue to record the relevant information and results on the data sheet as you go. Keep accurate records of what you have done. Do *not* enter all of your data after you complete the project.

8 When you have identified your organism, use *Bergey's Manual* or another standard reference to find one more test to run for confirmation. The confirmatory test does not have to separate the final organisms on the flowchart. It only has to be a test for which you know the result and that you have not run already as part of the identification process. Record this test on the data sheet and identify it as the confirmatory test. Record the *expected* result on the comments line. After incubation, note whether the organism's result matches or differs from the expected result.

9 After the confirmation test, write the organism's name on the back of the data sheet under "My Unknown is" and check with your instructor to see if you are correct. If you are, congratulations! If you are not, your instructor will advise you as to which test(s) gave "incorrect" results, by writing the test name(s) in the "rerun" space next to your identification. These should be rerun with appropriate controls from your school's inventory of organisms. Record the test(s) and control(s) on your data sheet. Checking the results with controls and your unknown will indicate if the "incorrect" test result was truly incorrect when previously run or if the strain of organism your school is using doesn't match the majority of strains for that test result.

[2] This flowchart can be used only with the organisms listed in Table 7-8.



7.26 Identification Flowchart for Gram-positive Rods ♦ See the appropriate exercises for instructions on how to run each test.

(Compiled from sources listed in References)

References

Brown-Elliot, Barbara A, and Richard J. Wallace, Jr. Chap. 30 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Collins, M. D. and C. S. Cummins. Section 15 in *Bergey's Manual of Systematic Bacteriology*, Vol. 2. John G. Holt, ed. Baltimore: Lippincott Williams & Wilkins, 1986.

Funke, Guido and Kathryn A. Bernard. Chap. 34 in *Manual of Clinical Microbiology*, 9th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Marie Louise Landry, and Michael A. Pfaller, eds. Washington, DC: ASM, 2007.

Hammes, Walter P. and Christian Hertel. Pages 465–511 in *Bergey's Manual of Systematic Bacteriology*, 2nd ed., Vol. 3, The Firmicutes. New York: Springer, 2009.

Logan, Niall A. and Paul De Vos. Pages 21–128 in *Bergey's Manual of Systematic Bacteriology,* 2nd ed., Vol. 3, The Firmicutes. New York: Springer, 2009.

Logan, Niall A., Alex R. Hoffmaster, Sean V. Shadomy, and Kendra E. Stauffer. Chap. 24 in *Manual of Clinical Microbiology*, 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

MacFaddin, Jean F. *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2000.

Pfyffer, Gaby E. and Frantiska Palicova. Chap. 28 in *Manual of Clinical Microbiology*, 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Rainey, Fred A., Becky Jo Hollen, and Alanna Small. Pages 738–828 in *Bergey's Manual of Systematic Bacteriology*, 2nd ed., Vol. 3, The Firmicutes. New York: Springer, 2009.

Stevens, Dennis L., Amy E. Bryant, Anja Berger, and Christoph von Eichel-Streiber. Chap. 50 in *Manual of Clinical Microbiology*, 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

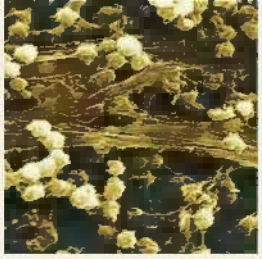
Wayne, Lawrence G., and George P. Kubrica. Section 15 in *Bergey's Manual of Systematic Bacteriology*, Vol. 2. John G. Holt, ed. Baltimore: Lippincott Williams & Wilkins, 1986.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 7-9

Identification of Gram-positive Rods

ISOLATION PROCEDURE

Unknown Number _____

Record all activities associated with isolation of your organisms—from mixed culture to pure culture. Always include the date, source of inoculum, destination, type of inoculation, incubation temperature, and any other relevant information. Also make note of transfers made to keep your pure culture fresh. (This log must be kept current.)

Preliminary Observations

Colony Morphology (include medium, incubation temperature, and differences between aerobic and anaerobic growth, if applicable)

Gram stain _____ Cellular morphology, arrangement, and dimensions _____

Differential Tests

Begin recording with your catalase result. This log must be kept current.

Record the Identification Chart you are using. If you change charts, indicate the figure number of the new charts and the date of the change.

Test #1: Date Begun: Date Read: Result:

Comments:

Test #2: Date Begun: Date Read: Result:

Comments:

Test #3: Date Begun: Date Read: Result:

Comments:

Test #4: Date Begun: Date Read: Result:

Comments:

Test #5: Date Begun: Date Read: Result:

Comments:

Test #6: Date Begun: Date Read: Result:

Comments:

Test #7: Date Begun: Date Read: Result:

Comments:

Test #8: Date Begun: Date Read: Result:

Comments:

Test #9: Date Begun: Date Read: Result:

Comments:

Test #10: Date Begun: Date Read: Result:

Comments:

Test #11: Date Begun: Date Read: Result:

Comments:

Test #12: Date Begun: Date Read: Result:

Comments:

My Unknown is: Rerun[1]:

Rerun:

Rerun:

[1] Your instructor will write what tests to rerun in this space if you misidentify your unknown.

SECTION 8

Environmental Microbiology

The topic of environmental microbiology encompasses exploring the microbial world around us to discover its diversity in water, soil, and many other habitats, as well as testing the environment (e.g., water) for public health reasons. You will do both in this section.

Section 8 begins with a fun exercise in which a mud culture is produced and then followed over the course of several weeks in the attempt to identify characteristic environmental microbes. This Winogradsky column will also serve as a source of organisms for subsequent labs. A mature column will produce beautiful colors (by comparison) out of dull, old mud!

The next four exercises (8-2 through 8-5) illustrate various aspects of the nitrogen cycle. Not only is it an important biogeochemical cycle, it is largely driven by microbial activity.

Following the nitrogen cycle, are three exercises (8-6 through 8-8) illustrating the sulfur cycle. It, too, relies heavily on microbial activity. It is in these exercises that you will harvest what you have grown in your Winogradsky column.

Exercise 8-9 illustrates bioluminescence, an interesting phenomenon exhibited by some bacteria. It is followed by an exercise in which you will grow microbes on a microscope slide inserted into soil for several days. Then, with careful removal and a special stain, you will observe the spatial relationships of the soil microbes you have cultivated. Exercise 8-11 allows you to use selective media and assess the microbial composition of a soil sample.

The last two exercises (8-12 and 8-13) are two different methods used by public health departments to test water for potability.

A Word about Trophic Groups

Understanding how bacteria make their living (i.e., where they get their nutrition and energy, whether they use organic or inorganic molecules for their purposes, where they live in relation to available oxygen, their optimum temperature, pH, and osmotic conditions) is fundamental to microbiology. In Section 2, we discussed major environmental factors that affect growth, but we did not talk about **trophic groups**. Trophic groups are categories designed by microbiologists to differentiate organisms based on their energy, electron, and carbon requirements.

It is probably obvious how important energy and carbon are to survival, but perhaps not so obvious why electrons are important. In many cases, organisms get their energy in the form of electrons stripped away from chemicals. For example, heterotrophs use organic compounds as a source of carbon, but they may also use those same compounds as an energy source by removing electrons and the energy they possess. An electron source is also important for organisms that use light energy, because it is the low energy electrons that are energized by light to a high enough potential energy that they can be used to reduce CO_2. Once on the carbon, those electrons must be replaced or the process grinds to a halt.

As you may have already predicted, there are terms assigned to the various categories. Undoubtedly, you have seen them. These are the words that always end with the suffix "-troph" or "-trophic," such as "autotroph," "heterotroph," or "phototroph."

The suffix, derived from the Greek word *trophe*, literally means "nutrition," but is used, slightly more broadly, to describe how an organism obtains its nutrition (carbon), electrons, and energy. Table 8-1 lists the trophic groups and the root words used to describe them. These roots are frequently combined to more fully describe an organism's requirements. For example, an organism that gets its energy from sunlight and fixes CO_2 as a source of carbon would be called a "photoautotroph." An organism that gets energy from the sun, but gets its carbon from organic molecules is called a "photoheterotroph." An organism that gets energy from inorganic molecules and fixes CO_2 is a "chemoautotroph." All combinations are represented by microbial species, but some are more common than others.

Unfortunately, stringing together all three terms can be a little cumbersome, so for example, you probably will never see the term chemoorganoheterotroph, although it clearly describes the sources of energy, electrons, and carbon needed by an organism. Using all three roots is usually redundant because an organism that uses organic molecules for a carbon source will also use those same organic molecules for energy by removing the electrons. Therefore, you are more likely to see the terms chemoorganotroph or chemoheterotroph. The same applies to chemolithoautotroph. Because it is able to use CO_2 as its carbon source, it is not likely to rely on organic molecules for electrons (organotroph), thus making it more efficient to simply call it a "chemoautotroph" or a "chemolithotroph."

Additionally, many descriptions do not include (or need) a comprehensive term such as chemolithoautotroph if they are meant only to describe one aspect of an organism's metabolism. For example, if the intent is to describe only that the organism uses CO_2 for its carbon needs, it may be called simply "autotrophic." If the intent is to say that the organism uses chemicals for energy rather than light, it may be called "chemotrophic."

In your reading, expect to see shortened versions of the terms. Experience has shown that students (and we were students!) have a difficult time learning and applying these terms because we all have been conditioned to think only in terms of plants (photoautotrophs) and animals (chemoheterotrophs). Whoever would have thought that nature would produce something so totally "novel" as a chemoautotroph or photoheterotroph! But by the time you complete these first few exercises, we anticipate that you will have become a trophic expert! ◆

TABLE **8-1** **Trophic Groups**

Energy Source	Descriptive Root	Carbon Source	Descriptive Root	Electron Source	Descriptive Root
Light	Photo-	CO_2	Auto-	Inorganic chemicals	Litho-
Chemical	Chemo-	Organic chemicals	Hetero-	Organic chemicals	Organo-

Winogradsky Column

EXERCISE 8-1

Theory

The Winogradsky column bears the name of its developer, Sergei Winogradsky (1856–1953), a Russian microbiologist and pioneer in microbial ecology. He studied sulfur bacteria because of their ease of handling and cultivation, and then moved on to bacteria associated with the nitrogen cycle. One of his major discoveries was finding microorganisms (*Beggiatoa*) capable of the unheard of type of metabolism that came to be known as chemolithotrophic autotrophy. Until he made his discovery, only photoautotrophs—those performing plant photosynthesis—were known to be autotrophs.

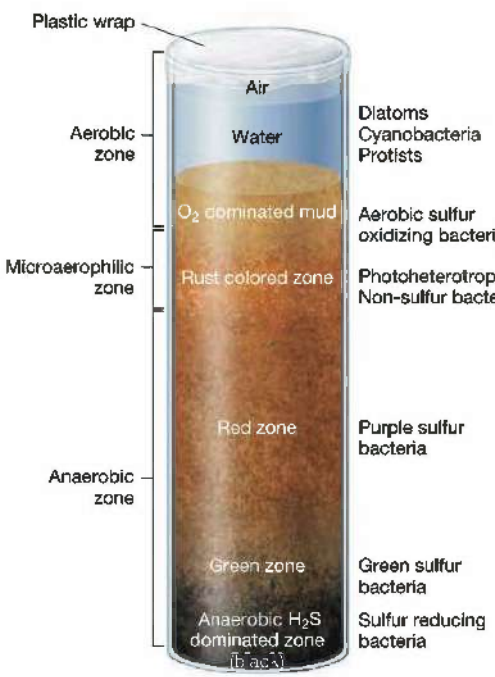
As a result of his work and the work of others, metabolic categories of microorganisms have been identified based on their carbon, energy, and electron sources. These were discussed in the sidebar A Word about Trophic Groups on page 582. Note that in practice, terms are combined to describe the organism more fully.

Winogradsky first used "his" column in the late 19th century. It was (and is) used as a convenient laboratory source to supply for study a variety of **anaerobic, microaerophilic,** and **aerobic** bacteria, including purple nonsulfur bacteria, purple sulfur bacteria, green sulfur bacteria, chemoheterotrophs, and many others (Fig. 8.1).

The basis for the Winogradsky column is threefold. The first two factors involve opposing gradients that impact the types of organisms that can grow. The first is the oxygen gradient, which gets more and more anaerobic toward the bottom. As a result, obligate aerobes, microaerophiles, facultative anaerobes, and obligate aerobes are found in different locations in the column. The second is the H_2S gradient, which runs opposite in direction to the O_2 gradient. The third factor is the diffuse light shined upon the column. This promotes growth of phototrophic organisms at levels where they are adapted to the opposing gradients of O_2 and H_2S. These layers of phototrophs occur in natural ecosystems but are extremely thin because light does not penetrate mud sediments very far. But with the transparent column, thicker layers develop, which are more easily sampled for cultures.

Application

The Winogradsky column is a method for growing a variety of microbes with uniquely microbial metabolic abilities. Bacterial photoautotrophs, chemolithotrophs, and photoheterotrophs may be found in a mature column. And more "typical" chemoheterotrophs and photoautotrophs also are likely to be found. A mature Winogradsky column is a good source for studying these organisms in the laboratory.



8.1 Artist's Rendition of a Winogradsky Column ♦ What you put into a Winogradsky column dictates what you grow. Any well-constructed column has an oxygen gradient from top to bottom, with the aerobic zone penetrating perhaps only as much as 20% of the total depth. The remaining portion of the mud column becomes progressively more anaerobic. The different amounts of oxygen lead to layering of microbial communities adapted to that specific environment. This illustration is a generalized picture of the layering that you might see in a mature column. (The column often produces intermixed patches rather than distinct layers.) Starting at the top and working downward, the layers are: air, water (containing algae and cyanobacteria), aerobic mud (sulfur oxidizing bacteria), microaerophilic mud (non-sulfur photosynthetic bacteria), red/purple zone (purple sulfur photosynthetic bacteria), green zone (green sulfur photosynthetic bacteria), black anaerobic zone (sulfur reducing bacteria).

In This Exercise

Using this procedure, you will produce an enrichment culture for growing aquatic sediment microorganisms with various metabolic abilities. In Exercises 8-6 through 8-8 you will be taking samples from the column for examination.

✓ Materials

Per Student Group

- □ Dirt or mud (approximately 250 g) from an aquatic source—to be collected by each student group and brought to lab
- □ Gloves
- □ One glass or plastic column, about 30 cm tall and 8 cm in diameter (available from biological supply houses)
- □ Calcium carbonate, 5 g
- □ Calcium sulfate, 5 g
- □ Shredded newspaper (or other cheap paper), 10 g
- □ Pan or bowl to hold the contents during mixing
- □ Spoon or spatula for mixing
- □ dH_2O
- □ Aluminum foil sheet
- □ Plastic wrap or Parafilm
- □ Rubber band
- □ Light source (a north-facing window sill or lamp that produces "natural" light)

8

Procedure

Note: This procedure is very forgiving with respect to amounts. Try to approximate the suggested amounts as much as you can, but don't fret if you are off by a little bit. This isn't brain surgery. But DO wear gloves.

1. Remove large chunks, rocks, and debris from the mud/dirt sample.
2. To about 125 g mud in a mixing pan, add 5 g of calcium carbonate and 5 g of calcium sulfate. Add water (if it isn't already in the sample). Mix thoroughly.
3. Add the 10 g of shredded paper and mix again.
4. Add this enriched slurry to the column to a depth of 3 cm. Pack it firmly with a stirring rod so no air spaces remain. *Removing air spaces is probably the most important step.*
5. Add the remaining unenriched mud to a level about 5 cm from the top of the column and pack again.
6. Add water to a depth of 2 cm–3 cm on top of the mud. Let it sit for about 30 minutes. If there is more water than you put in, remove the excess. If the water level has dropped, add more until you have the correct depth. There should be about 2–3 cm of air space above the water at this point.
7. Cover the top with foil or plastic wrap held on with a rubber band.
8. Incubate for 6 or 7 weeks in indirect sunlight at room temperature (Fig. 8.2).
9. Check the column weekly to watch its development (Fig. 8.3).
10. Record your observations on the data sheet, page 587.

Weeks Later

Your instructor will tell you how you will use the column as a source of samples in Exercises 8-6 through 8-8.

8.2 Freshly Made Winogradsky Column ◆ The black layer comprising the majority of the column is the unenriched mud. The lighter gray area at the bottom contains mud, $CaCO_3$, $CaSO_4$, and shredded paper mixed into a slurry. Note the absence of air spaces.

8.3 Mature Winogradsky Column at Eight Weeks ♦ Notice the layers and colors! Also notice that the layers are not as well defined as in Fig. 8.1. In fact, some look mixed (e.g., the rust and red portions appear mixed in some regions). But the dark, anaerobic zone above the whitish layer at the bottom is well defined. The remainder is—pardon the expression—clear as mud.

References

Atlas, Ronald M. and Richard Bartha. *Microbial Ecology—Fundamentals and Applications*, 4th ed. Menlo Park, CA: Addison Wesley Longman, 1998.

Lens, Piet, Marcus Vallero, and Look Hulshoff Pol. Chap. 38 in *The Desk Encyclopedia of Microbiology*. Moselio Schaechter, ed. San Diego, CA: Elsevier Academic Press, 2004.

Madigan, Michael T., John M. Martinko, Paul V. Dunlap, and David P. Clark. *Brock Biology of Microorganisms*, 12th ed. San Francisco, CA: Pearson Benjamin Cummings, 2009.

Perry, Jerome J., James T. Staley, and Stephen Lory. *Microbial Life*. Sunderland, MA: Sinauer Associates, Publishers, 2002.

Varnam, Alan H., and Malcom G. Evans. *Environmental Microbiology*. Washington, DC: ASM Press, 2000.

Willey, Joanne M., Linda M. Sherwood, and Christopher J. Woolverton. *Prescott's Principles of Microbiology*. Boston: McGraw-Hill Higher Education, 2009.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-1

Winogradsky Column

OBSERVATIONS AND INTERPRETATIONS

Soil Sample Site: _____

1 Record your observations in the table below.

Week	**Observations** (include distinctive color development and its level in the column)
1	
2	
3	
4	
5	
6	
7	
8	

QUESTIONS

1 *What is/are the carbon source(s) in the Winogradsky column?*

2 *Based on the metabolic reaction, fill in the Mode of Metabolism column. Use the definitions under "Theory" as a guide, and the aerobic heterotroph as an example.* **Note:** *hν represents light and CH_2O represents organic carbon. Most reactions are simplified to show the "essence" of the process without excessive detail.*

Organism	Aerotolerance	Mode of Metabolism (e.g., phototroph, autotroph, chemotroph, etc.)	Metabolic Reaction
Aerobic heterotrophs	Obligate aerobe	Chemoheterotrophic	$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$
Cyanobacteria	Aerobe		$6CO_2 + 6H_2O \xrightarrow{h\nu} C_6H_{12}O_6 + 6O_2$
H_2S oxidizers (*Beggiatoa*)	Microaerophile		$CO_2 + H_2S \longrightarrow CH_2O + S^0$
Purple non-sulfur bacteria	Microaerophilic anaerobe		$CH_2O \longrightarrow CO_2 + H_2$, and $CO_2 + H_2 \xrightarrow{h\nu} CH_2O$
Purple sulfur bacteria	Anaerobe		$CO_2 + H_2S \xrightarrow{h\nu} CH_2O + S^0$
Green sulfur bacteria	Anaerobe		$CO_2 + H_2S \xrightarrow{h\nu} CH_2O + S^0$
Sulfate reducers	Anaerobe		$CH_2O + SO_4^{2-} \longrightarrow CO_2 + H_2S$

3 *Why is $CaSO_4$ mixed into the lowermost, enriched slurry when making the column?*

4 *Why does H_2S accumulate in the most anaerobic region of the column?*

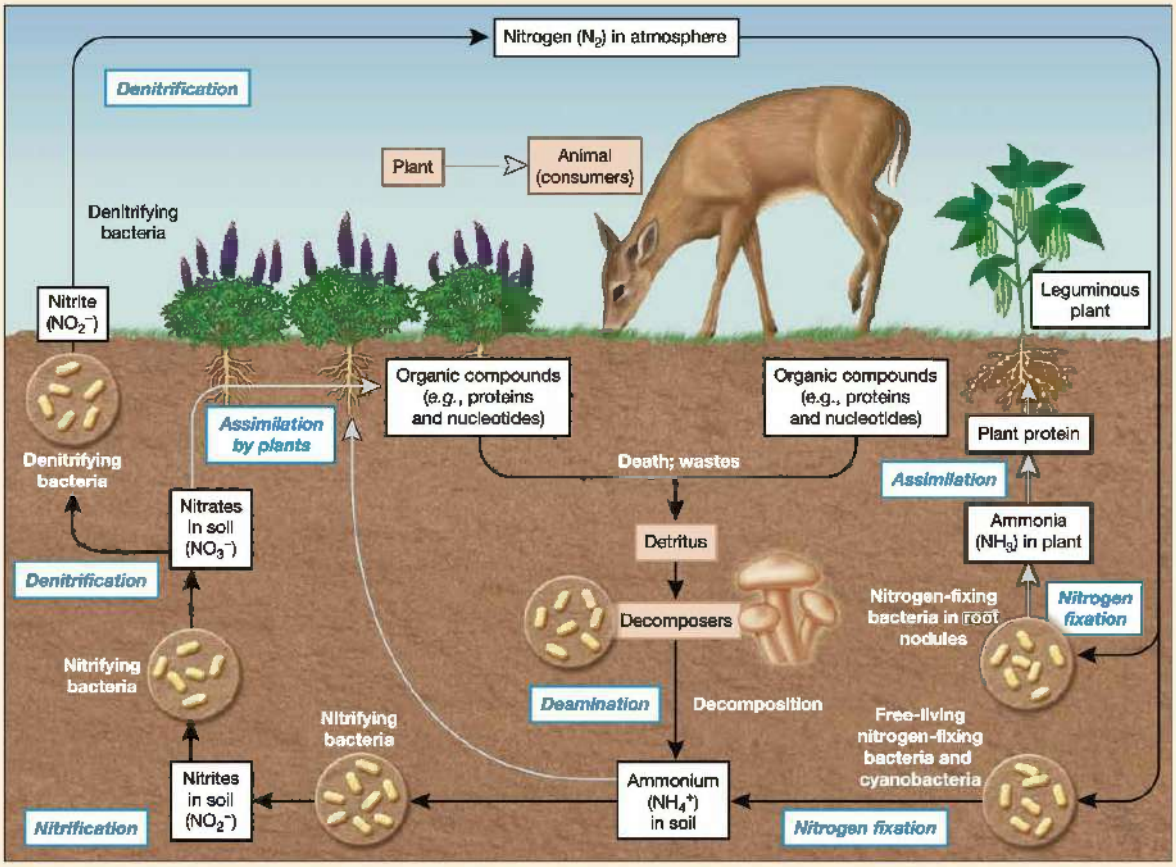
5 *In what way does photosynthesis differ between cyanobacteria and the green and purple sulfur bacteria?*

6 *Why can chemoheterotrophs grow throughout the column?*

Introduction to the Nitrogen Cycle

Biogeochemical cycles, such as the carbon cycle, the nitrogen cycle, and the sulfur cycle, are characterized by **environmental phases**, in which the element is not incorporated into an organism, and an **organismal phase**, in which it is. All are important, but because the nitrogen cycle has so many parts in which bacteria participate, it will be discussed here. It will be helpful for you to look at Figure 8.4 as you read the following.

Organisms require nitrogen as a component of the 20 amino acids, purine and pyrimidine nucleotides, and other compounds. These organic forms of nitrogen do not occur outside cells. Most of the air (approximately 80%) is nitrogen gas (N_2), but this is a form of nitrogen that is not usable by all organisms except **nitrogen-fixing bacteria.** Some nitrogen-fixing bacteria (e.g., *Rhizobium*) are symbionts of certain legumes and form nodules in their roots. Other nitrogen-fixing bacteria are free-living in the soil (e.g., *Azotobacter*) or water (e.g., *Nostoc*). The process of nitrogen fixation is highly endergonic, requiring about 16 moles of ATP per mole of N_2 reduced to ammonia (NH_3).



8.4 Nitrogen Cycle ◆ There are three sets of information shown in this nitrogen cycle diagram: the nitrogenous compounds (in white boxes and black type), the process in which they are used (white boxes, blue type), and a general name for the organisms doing each (white text). Black arrows are microbial processes; gray arrows are macroorganismal processes. Further (but not labeled), the nitrogen cycle involves chemoheterotrophs (both aerobic and anaerobic respirers) and chemolithotrophs. See text for details.

Because nitrogen fixation requires so much energy, the **nitrogenase** enzyme is not active if other forms of nitrogen are available. In addition, nitrogenase is inactivated by oxygen, so a variety of mechanisms have evolved to protect it from oxygen. You will examine N-fixation in Exercise 8-2.

Some chemolithotrophic bacteria are capable of **nitrification**, in which ammonium ion acts as an electron and energy source when it is oxidized to nitrite, and then to nitrate. Organisms such as *Nitrosomonas* oxidize ammonium to nitrite; then, other nitrifiying organisms, such as *Nitrobacter*, continue the oxidation of nitrite to nitrate. The same organisms do not do both. You will examine nitrification in Exercise 8-3.

Once nitrogen gas has been reduced to ammonium ion, it becomes available for **assimilation** into amino acids by green plants and other bacteria. It then enters the food chain as various forms of organic nitrogen, the only form of nitrogen animals can use. **Oxidative deamination** of amino acids during heterotrophic catabolism (including decomposition by bacteria and fungi) results in the formation of ammonium ion once again. You will examine this process of ammonification in Exercise 8-4.

Denitrifying bacteria, which are heterotrophs, use nitrate as the final electron acceptor of **anaerobic respiration**. The end products of nitrate reduction include nitrogen gas, nitrite, and ammonium ion. You will examine denitrification in Exercise 8-5. ◆

EXERCISE
8-2

Nitrogen Fixation

Theory

Fixation of atmospheric nitrogen (N_2) occurs through the following reaction:

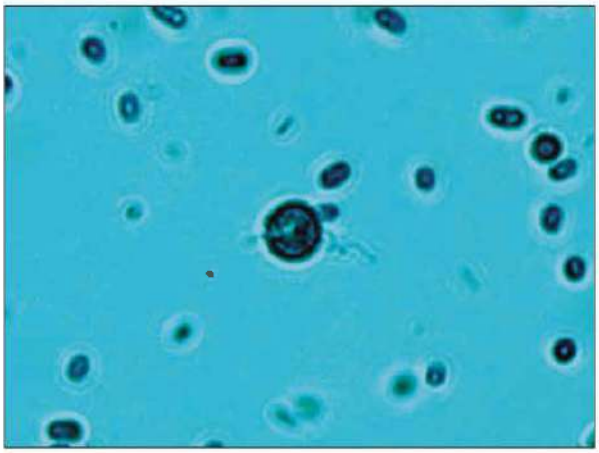
$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

This is a highly endergonic process and is performed by only a few groups of bacteria. Among terrestrial bacteria, *Azotobacter* (a β-proteobacterium) and *Rhizobium* (an α-proteobacterium) usually are put forth as examples.

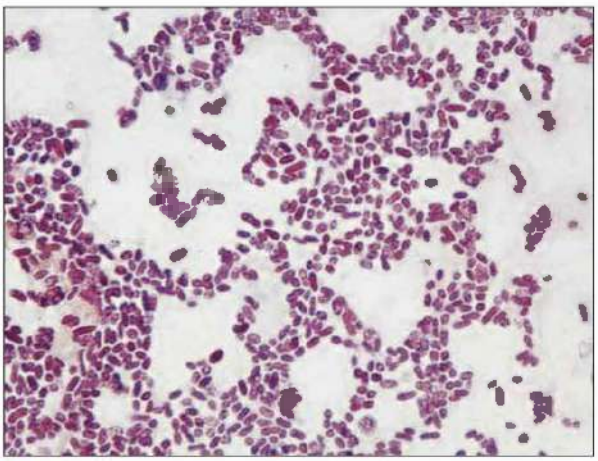
Azotobacter (Fig. 8.5) is a common, free-living, aerobic Gram-negative rod that fixes N_2 in the soil. N-fixation is inhibited by oxygen because it inhibits the **nitrogenase** that is responsible for fixation. The ability of *Azotobacter* to fix nitrogen aerobically is attributable to a number of complex, species-specific mechanisms that are beyond the scope of this book. *Azotobacter* also has the ability to form resting vegetative cells called **cysts** when environmental conditions are not favorable (Fig. 8.6). Although they perform somewhat the same function as bacterial endospores, they are different in that they are not as differentiated from the cell that formed them, nor are they as resistant to environmental factors such as desiccation and physical and chemical agents.

Rhizobium (Fig. 8.7) is a mutualistic symbiont of leguminous plants (members of Fabaceae: the Pea family). They rely on a supply of carbon compounds as electron donors from the plant host and, in return, supply the plant with ammonium, which can be assimilated into organic nitrogen (amino acids, nucleotides, etc.). *Rhizobium* and other symbionts are the major N-fixers on land.

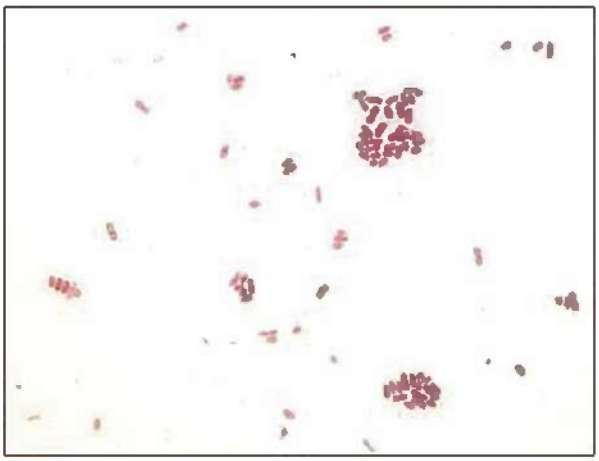
Rhizobium is a Gram-negative rod that enters the root and induces the formation of **root nodules** (Fig. 8.8), tumors that are the location of N-fixation. Once in the plant, the cells become irregular in shape and are referred to as **bacteroids** (Fig. 8.9). Their adaptation to fixing nitrogen aerobically is the presence of **leghemoglobin**,



8.6 *Azotobacter* Cyst Stain ♦ *Azotobacter* forms resting cells called cysts when the environment becomes unsuitable. They can be viewed with a wet mount stain.

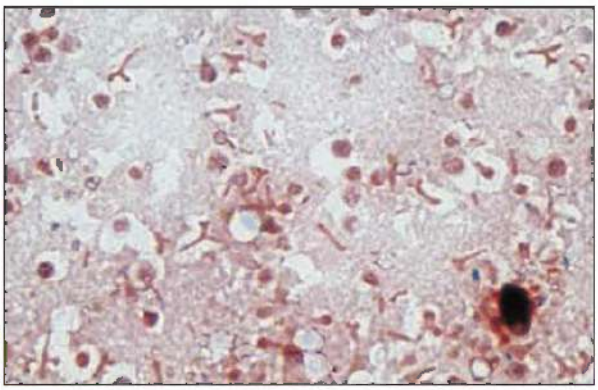


8.5 *Azotobacter* Gram Stain ♦ Plump, Gram-negative rods characterize the free-living, nitrogen-fixing *Azotobacter*. These cells were grown in culture.

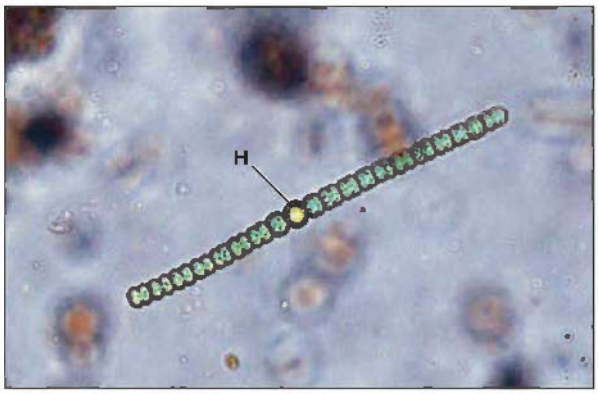


8.7 *Rhizobium* Gram Stain ♦ *Rhizobium* occurs naturally as a nitrogen-fixing symbiont of leguminous plants. This Gram stain was made from a culture.

8.8 Clover Root Nodules ◆ The roots of this small clover have been infected with *Rhizobium* that causes the formation of tumor-like root nodules (four are circled).



8.9 Root Nodule Section ◆ Symbiotic nitrogen-fixing bacteria, such as *Rhizobium*, induce tumor formation in the roots of certain legumes. Once in the nodule, infected cells become filled with differentiated forms of the bacterium called bacteroids. Uninfected cells are white in this preparation.

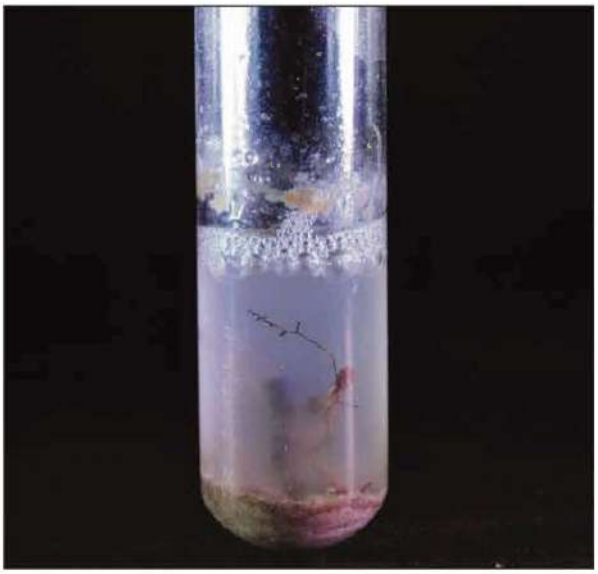


8.10 Heterocyst in *Anabaena* ◆ *Anabaena* is a filamentous cyanobacterium. Specialized, thick-walled cells called heterocysts (H) are involved in nitrogen fixation. The oxygen-sensitive nitrogenase enzyme is protected from oxygen in these cells because they lack Photosystem II, which produces oxygen from water during photolysis, and the thick wall apparently limits oxygen diffusion from the environment.

which, like our own hemoglobin, binds free oxygen. Interestingly, its production is induced through the combined efforts of the host plant and *Rhizobium*. Leghemoglobin is so effective at moderating O_2 concentrations in the nodule that the bound form is estimated to outnumber the free form 10,000:1!

In aquatic environments, cyanobacteria are the main nitrogen fixers. An interesting example is the filamentous species *Anabaena* (Fig. 8.10). Cyanobacteria perform photosynthesis using the same process as green plants. That is, they convert CO_2 and H_2O to carbohydrate and O_2. Because N-fixation is inhibited by oxygen, they restrict the process to thick-walled cells called **heterocysts**, which lack Photosystem II, the oxygen-producing component of photosynthesis (Fig. 8.10). Further, they are thick-walled, which restricts entry of O_2 by diffusion. Heterocysts rely on physical connection with adjacent cells to supply reducing power for N-fixation and, in return, supply the other cells of the filament with organic nitrogen compounds.

Nitrogen-free broth is an enrichment medium lacking a nitrogen source other than the air. Therefore, only N-fixing organisms can survive in this broth. We will be testing for free-living N-fixers, such as *Azotobacter*. When *Azotobacter* is present, you will see a pellicle at the surface of the medium, and often gas bubbles (Fig. 8.11). Whether you see a pellicle or not, N-fixers will produce ammonia and you will perform a qualitative test for its presence.



8.11 Nitrogen-Free Broth ◆ This nitrogen-free broth was inoculated with a soil sample. Because no nitrogen is present in the medium, only N-fixers can grow. Notice the bubbles and pellicle at the surface.

Application

Soil fertility is determined largely by the rate and amount of N-fixation. This technique provides soil microbiologists with a mechanism to perform an assay for soil N-fixing organisms. This exercise may be combined with any or all of Exercises 8-3, 8-4, and 8-5.

In This Exercise

You will bring a soil sample to lab and incubate a portion of it in nitrogen-free broth. After incubation, you have several options.

1. To see that organisms can grow in the selective nitrogen-free broth—meaning that you have isolated a community of N-fixers. If so, you will perform a quantitative test for ammonia, the product of N-fixation.
2. To examine the culture for a pellicle, a sure sign that *Azotobacter* is present.
3. To perform a Gram stain on the broth.
4. To perform an *Azotobacter* cyst stain and a capsule stain on the pellicle.

Your choice(s) likely will be dictated by lab time and availability of resources.

✓ Materials

Per Student

- ☐ One capped 25 mL tube of soil from a location of your choice (tubes should be handed out the previous lab). This soil sample can be used for the other exercises relating to the nitrogen cycle (Exercises 8-3 through 8-5).
- ☐ Two nitrogen-free broth tubes (5 mL–8 mL)
- ☐ Two clear plastic microtubes
- ☐ Two 15 × 100 test tubes
- ☐ Four transfer pipettes
- ☐ Three microscope slides
- ☐ Gloves

Per Student Group

- ☐ API Freshwater Master Test Kit[1]
  - Ammonia reagents #1 and #2
- ☐ Microfuge
- ☐ Digital balance and weigh boat
- ☐ Spatula
- ☐ (Optional) coarse screen
- ☐ (Optional) Gram stain kit
- ☐ (Optional) *Azotobacter* stain

Medium and Stain Recipes

Nitrogen-Free Broth

• Glucose	10 g
• $CaCO_3$	5 g
• $CaCl_2$	0.1 g
• KH_2PO_4	0.1 g
• K_2HPO_4	0.9 g
• $MgSO_4$	0.1 g
• $FeSO_4$	10 mg
• Na_2MoO_4	5 mg
• Distilled or deionized water	1 L

Azotobacter Cyst Stain

• Glacial acetic acid	8.5 mL
• Sodium sulfate	3.25 g
• Neutral red	200 mg
• Light green	200 mg
• Ethanol	50 mL
• Distilled or deionized water	100 mL

[1] API Freshwater Test Kit is available from Mars Fishcare North America, Inc. PO Box 218, Chalfont, PA 18914. 1-800-847-0659 or www.aquariumpharm.com.

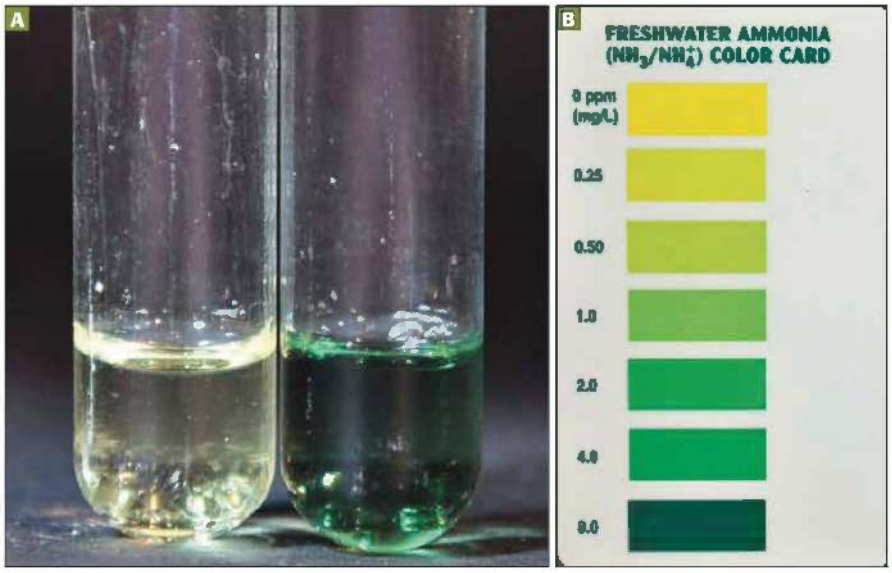
Procedure

Lab One

1. Obtain two nitrogen-free broth tubes.
2. Try to remove as many rocks and sticks as possible from the soil. Passing it through a screen is helpful.
3. Weigh 1 gram of soil.
4. Transfer the 1 g of soil to one NFB tube and label it "NFB-Sample" or "NFB" and the soil source. Leave the other uninoculated as a control and label it "NFB-Control."
5. Incubate both NFB tubes for up to 7 days at 25°– 30°C or until a pellicle forms on the surface.

Lab Two

1. Examine the inoculated tube for turbidity, which indicates you have isolated one or more N-fixing bacteria.
2. Examine the inoculated tube for a pellicle, which indicates that you likely have grown *Azotobacter* (Fig. 8.11).
3. (Optional) If a pellicle is present, perform a:
   a. Gram stain (see Exercise 3-7 for the procedure)
   b. Capsule stain (see Exercise 3-9)
   c. *Azotobacter* cyst stain by making a wet mount in the stain solution.
4. Use a transfer pipette to dispense 1.0 mL of your culture into a clear plastic microtube. Close the cap tightly. Label it "NFB-Culture."
5. With a fresh transfer pipette, put an *equal* volume of uninoculated nitrogen-free broth in the second clear plastic microtube. Close the cap tightly. Label it "NFB-Control."
6. Label both caps with your initials.
7. Spin both tubes for 2 minutes at high speed in a microfuge. Be sure to balance the microfuge by placing your tubes opposite one another.
8. With a transfer pipette, carefully draw the broth out of your sample without disturbing the pellet at the bottom, and transfer 0.6 mL to a clean glass test tube. Label this tube with your name and "NFB-Sample."
9. Using a fresh transfer pipette, remove 0.6 mL from the "NFB-Control" tube and transfer it to a clean glass test tube.
10. **Test for ammonia.** Test for ammonia using the reagents supplied.
    a. Add one drop of ammonia reagent #1 and one drop of ammonia reagent #2 to "NFB-Sample" and to "NFB-Control." ***Note***: these bottles should be held upside-down, but vertically, for the drops to be the correct volume.
    b. Mix the tubes for 5 seconds.
    c. Watch for color to develop in 5 minutes (Fig. 8.12A).
    d. Compare the color against the color card to determine the ammonia concentration in your soil sample (Fig. 8.12B). ***Note***: use the color card that came with the reagents for color comparison, not the photo in Figure 8.12B.
    e. Record your results on the data sheet, page 597.



8.12 Test for Ammonia ♦
(A) Nitrogen fixation results in the production of ammonia. Shown are results of a quantitative test for ammonia, with negative on the left and positive on the right. (B) The color card matches test result colors with approximate ammonia concentrations. This sample has approximately 8 mg/L of ammonia. (***Note:*** Use the actual color card, not this photograph, for comparison.)

References

Atlas, Ronald M. *Handbook of Media for Environmental Microbiology*, 2nd ed. Boca Raton, FL: CRC Press, 2005.

Atlas, Ronald M. and Richard Bartha. *Microbial Ecology—Fundamentals and Applications*, 4th ed. Menlo Park, CA: Addison Wesley Longman, 1998.

Kennedy, Christina, Paul Rudnick, Melanie L. MacDonald, and Thoyd Melton. *Genus III. Azotobacter* Beijerinck 1901, 567^{AL} in *Bergey's Manual of Systematic Bacteriology*, 2nd ed., Vol. Two, *The Proteobacteria—Part B The Gammaproteobacteria*. New York: Springer, 2005.

Madigan, Michael T., John M. Martinko, Paul V. Dunlap, and David P. Clark. *Brock Biology of Microorganisms*, 12th ed. San Francisco: Pearson Benjamin Cummings, 2009.

Microbiology Procedures: http://www.microbiologyprocedure.com/staining-methods-in-microbiology/stain-for-azotobacter-cysts.html

Varnam, Alan H. and Malcom G. Evans. *Environmental Microbiology*. Washington, DC: ASM Press, 2000.

Willey, Joanne M., Linda M. Sherwood, and Christopher J. Woolverton. *Prescott's Principles of Microbiology*. Boston: McGraw-Hill Higher Education, 2009.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-2

Nitrogen Fixation

OBSERVATIONS AND INTERPRETATIONS

Soil Sample Site: _____

1 Record your observations in the table below. Also record results from three classmates who worked with different soil samples.

Tube (include source of soil samples)	Matching Color on Card	Ammonia Concentration (mg/L or ppm)
Control		
Soil sample:		
Soil sample:		
Soil sample:		
Soil sample:		

2 If you did staining, record your observations in the table below. Include sketches and measurements of cell dimensions.

Organism	Gram Stain	Capsule Stain	Cyst Stain
Pellicle, probable *Azotobacter*			
Pellicle, other species			NA

QUESTIONS

1 *Consider the uninoculated tube.*

a. *Is it a positive or a negative control?*

b. *What information does the uninoculated control provide?*

2 *Without even looking at the organisms microscopically, how can we be certain that the organisms growing in nitrogen-free broth are N-fixers?*

3 *Why are N-fixers so important in food chains?*

4 *Besides plants, what other organisms can directly utilize fixed nitrogen? Using one of each pair of the terms auto/heterotroph and litho/organotroph, to which group do these other organisms belong?*

Nitrification: The Production of Nitrate

EXERCISE 8-3

Theory

Nitrification is the process of making **nitrate.** This is strictly a microbial process performed by **nitrifying bacteria** and is a two-step, exergonic process involving two different groups of aerobic **chemolithotrophs** (which also are **autotrophs** because they use the energy released to fix carbon dioxide into organic carbon).

First, bacteria, such as *Nitrosomonas*, oxidize ammonium (NH_4^+) to form nitrite (NO_2), as follows:

$$NH_4^+ + 1\frac{1}{2}\ O_2 \rightarrow NO_2^+ + H_2O + 2H^+$$

The second step involves different chemolithotrophs, such as *Nitrobacter*, that oxidize nitrite to nitrate, as follows:

$$NO_2^- + \frac{1}{2}\ O_2 \rightarrow NO_3^-$$

Clay particles in soil bind positively charged ions, whereas negatively charged ions are repelled and are more available for organismal use, because they are freely diffusible in the soil water. With that fact in mind, nitrification clearly is an important ecological process because it changes the charge on nitrogen from positive (in ammonium ion) to negative (nitrite and nitrate). Nitrate, along with ammonium, is a form of nitrogen that plants can absorb into their roots. Because of their solubility, however, nitrite and nitrate are readily leached from soil, making it less fertile.

Application

This exercise shows a simple way to determine the presence of aerobic chemolithotrophic bacteria that (1) oxidize ammonia to nitrite, and (2) oxidize nitrite to nitrate. This exercise may be combined with any or all of Exercises 8-2, 8-4, and 8-5.

In This Exercise

You will bring a soil sample to class and check it for activity of microbial ammonia oxidizers that produce nitrite and nitrite oxidizers that produce nitrate.

✓ Materials

Per Student

- ☐ One capped 25 mL tube of soil from a location of your choice (tubes should be handed out the previous lab). This soil sample can be used for the other exercises relating to the nitrogen cycle (Exercises 8-2, 8-4, and 8-5).
- ☐ Two ammonium broth tubes (5 mL–8 mL)
- ☐ Five clear plastic microtubes
- ☐ Five 15 × 100 glass test tubes
- ☐ Six transfer pipettes
- ☐ Gloves

Per Student Group

- ☐ API Freshwater Master Test Kit[1]
  - Ammonia reagents #1 and #2
  - Nitrate reagents #1 and #2
  - Nitrite reagent
- ☐ Microfuge
- ☐ Digital balance and weigh boat
- ☐ Spatula
- ☐ (Optional) coarse screen

Medium Recipe

Ammonium Broth

• Ammonium sulfate	1.0 g
• $MgSO_4$	0.5 g
• NaCl	2.0 g
• K_2HPO_4	1.0 g
• $CaCO_3$	10.0 g
• $FeCl_3$	trace
• Distilled or deionized water	1 L

Procedure

Lab One

1. Obtain two ammonium broth tubes.
2. Try to remove as many rocks and sticks as possible from the soil. Passing it through a screen is helpful.
3. Weigh 1 gram of soil.
4. Transfer the 1 g of soil to one ammonium broth tube. Label it "AB-Sample" or "AB" and the soil source. Leave the other uninoculated as a control. Label it "AB-Control."

[1] API Freshwater Test Kit is available from Mars Fishcare North America, Inc. PO Box 218, Chalfont, PA 18914. 1-800-847-0659 or www.aquariumpharm.com.

5 Incubate both AB tubes for up to 7 days at 25°–30°C.

Lab Two

1 Examine the inoculated tube for turbidity, which indicates that you have isolated one or more ammonia-oxidizing bacteria. If there is none, you may wish to continue incubation for a few days longer.

2 Use a transfer pipette to dispense 1.5 mL of your culture into two clear plastic microtubes. Close the caps tightly. Label one "NO_2 Sample" and the other "NO_3 Sample."

3 Transfer 1.5 mL of uninoculated ammonium broth to the other three clear plastic microtubes using a fresh transfer pipette. Label as follows: "AB-Control 1," "AB-Control 2," and "AB-Control 3."

4 Label the five caps with your initials.

5 Spin all tubes for 2 minutes at high speed in a microfuge. Be sure to balance the microfuge by placing your tubes opposite one another. You will have to combine with another student to make the balancing even, or you can use water to make the sixth tube.

6 Carefully draw 1 mL of broth out of each sample tube without disturbing the pellet at the bottom, and transfer the broth to two clean glass test tubes. Label these tubes with your name and "NO_2 Sample" and "NO_3 Sample."

7 Transfer 1 mL from "AB-Control 1" to a clean glass test tube. Label it "Control 1." (You may use the same transfer pipette for steps 8 and 9.)

8 Transfer 1 mL from "AB-Control 2" to a clean glass test tube. Label it "Control 2."

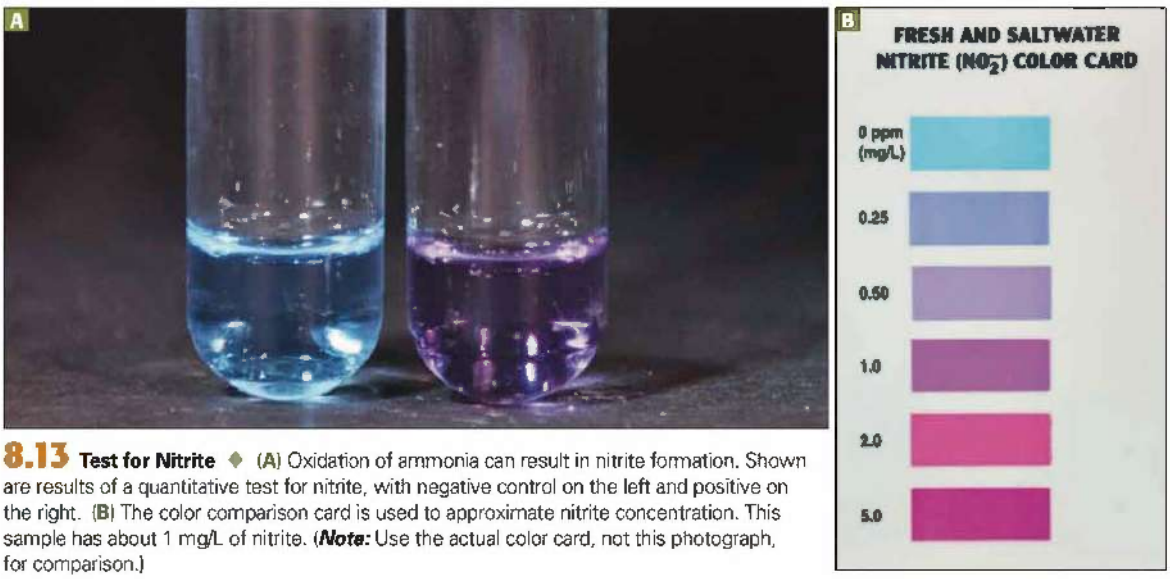
9 Transfer 1 mL from "AB-Control 3" to a clean glass test tube. Label it "Control 3."

10 **Test for nitrite.** Use the reagent supplied to perform the test for nitrite as follows:

- a Add one drop of nitrite reagent to "NO_2-Sample" and "Control 1." Mix for 5 seconds. ***Note:*** this bottle should be held upside-down, but vertically, for the drops to be the correct volume.
- b Watch for color to develop in 5 minutes (Fig. 8.13A).
- c Compare the color against the color card to determine the nitrite concentration in your soil sample (Fig. 8.13B).
- d Record your results on the data sheet, page 603.

11 **Test for nitrate.** Use the reagents supplied to perform the test for nitrate as follows:

- a Add two drops of nitrate reagent #1 to "NO_3-Sample" and to "Control 2." Mix for 5 seconds. Hold the bottle vertically when dispensing the two drops.
- b Shake nitrate reagent #2 for 30 seconds.
- c Holding the bottle vertically, add two drops of nitrate reagent #2 to "NO_3-Sample" and to "Control 2." Mix vigorously for 1 minute.
- d Watch for color to develop in 5 minutes (Fig. 8.14A).
- e Compare the color against the color card to determine the nitrite concentration in your soil sample (Fig. 8.14B).
- f Record your results on the data sheet, page 603.



8.13 Test for Nitrite ◆ (A) Oxidation of ammonia can result in nitrite formation. Shown are results of a quantitative test for nitrite, with negative control on the left and positive on the right. (B) The color comparison card is used to approximate nitrite concentration. This sample has about 1 mg/L of nitrite. (***Note:*** Use the actual color card, not this photograph, for comparison.)

12 Test for ammonia. Use the reagents supplied to test for ammonia as follows:

- **a** Holding the bottles vertically, add one drop of ammonia reagent #1 and one drop of ammonia reagent #2 to "Control 3." Mix for 5 seconds.
- **b** Color should develop in 5 minutes (Fig. 8.15A).
- **c** Compare the color against the color card to determine the ammonia concentration in your soil sample (Fig. 8.15B).
- **d** Record your results on the data sheet.

References

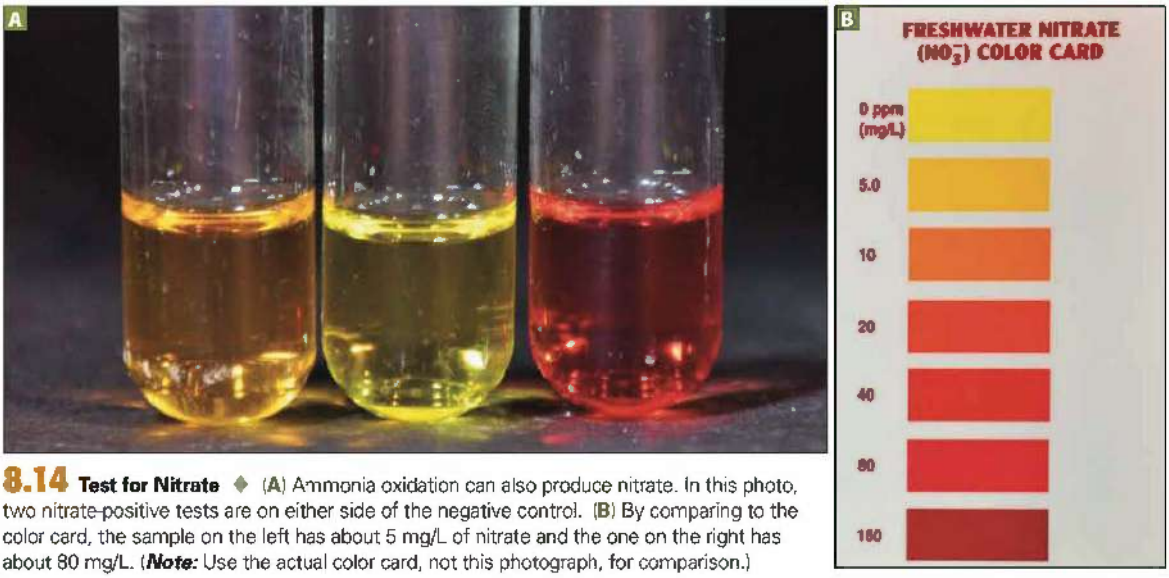
Atlas, Ronald and Richard Bartha. *Microbial Ecology—Fundamentals and Applications*, 4th ed. Menlo Park, CA: Addison Wesley Longman, 1998.

Bartholomew, James W. *Laboratory Textbook and Exercises in Microbiology*. Dubuque, IA: Wm. C. Brown Company Publishers, 1967.

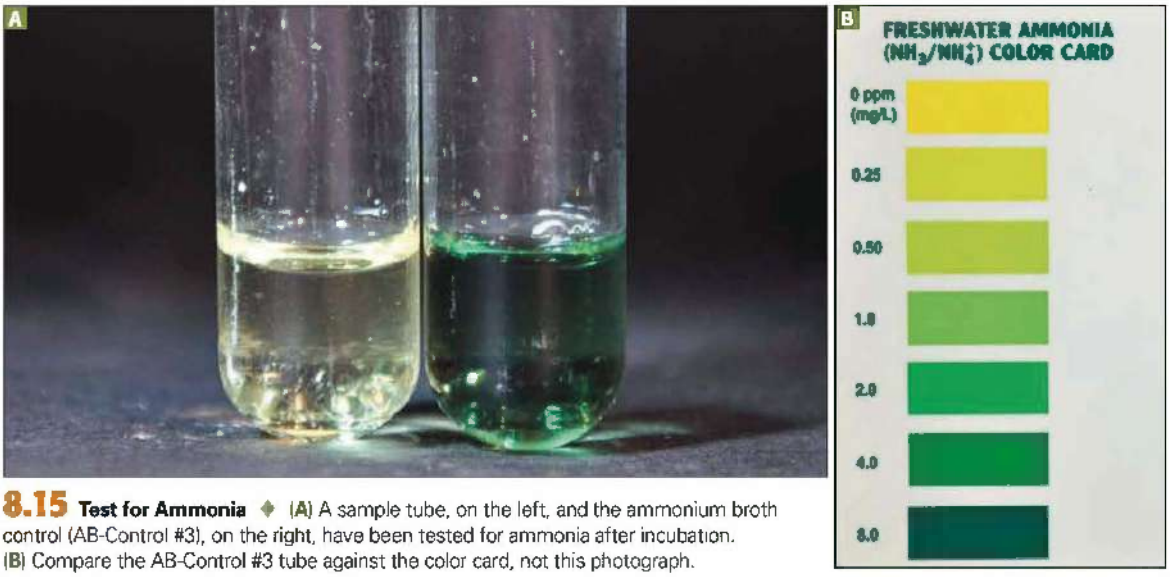
Varnam, Alan H. and Malcom G. Evans. *Environmental Microbiology*. Washington, DC: ASM Press, 2000.

White, David. *The Physiology and Biochemistry of Prokaryotes*, 2nd ed. New York and Oxford: Oxford University Press, 2000.

Willey, Joanne M., Linda M. Sherwood, and Christopher J. Woolverton. *Prescott's Principles of Microbiology*. Boston: McGraw-Hill Higher Education, 2009.



8.14 Test for Nitrate ♦ (A) Ammonia oxidation can also produce nitrate. In this photo, two nitrate-positive tests are on either side of the negative control. (B) By comparing to the color card, the sample on the left has about 5 mg/L of nitrate and the one on the right has about 80 mg/L. (***Note:*** Use the actual color card, not this photograph, for comparison.)



8.15 Test for Ammonia ♦ (A) A sample tube, on the left, and the ammonium broth control (AB-Control #3), on the right, have been tested for ammonia after incubation. (B) Compare the AB-Control #3 tube against the color card, not this photograph.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-3

Nitrification: The Production of Nitrate

OBSERVATIONS AND INTERPRETATIONS

Soil Sample Site: _____

1 Record your results in the table below. Also record results from three classmates who worked with different soil.

Tube (include soil sample sources)	Matching Color on Card	Nitrite Concentration (mg/L or ppm)	Nitrate Concentration (mg/L or ppm)	Ammonia Concentration (mg/L or ppm)
AB–Control 1			NA	NA
AB–Control 2		NA		NA
Soil sample:	NO_2			NA
	NO_3			
Soil sample:	NO_2			NA
	NO_3			
Soil sample:	NO_2			NA
	NO_3			
Soil sample:	NO_2			NA
	NO_3			
AB–Control 3		NA	NA	

QUESTIONS

1 *Consider AB-Control 1*

a. *Is it a positive or a negative control?*

b. *What information is provided by AB-Control 1?*

2 *Consider AB-Control 2.*

a. *Is it a positive or a negative control?*

b. *What information is provided by AB-Control 2?*

3 *Consider AB-Control 3.*

a. *Is it a positive or a negative control?*

b. *What information is provided by AB-Control 3?*

4 *Why might the results you obtain for your soil show low amounts of nitrite and high amounts of nitrate?*

5 *Why might the results you obtain for your soil show high amounts of nitrite and low amounts of nitrate?*

Ammonification

EXERCISE 8-4

Theory

In an ecosystem, much of the nitrogen is tied up in organic molecules. No organism lives forever, however, or is immune from producing wastes. **Ammonification** is a consequence of decomposition of organic nitrogen from dead animal and plant protein, and nitrogenous wastes (e.g., urea) from animals, and is a byproduct of amino acid **deamination** (a step in amino acid catabolism) in cells. Many bacteria and fungi are capable of ammonification. The ammonia so produced can be recycled through uptake by plants, or it can be oxidized by ammonia oxidizing bacteria (see Exercise 8-3).

Deamination of an aminio acid occurs as follows:

Application

Ammonia is an important soil component because of its availability to plants as a source of nitrogen and their ability to incorporate it into organic nitrogen again. This test allows for rapid determination of ammonia production in soil. This exercise may be combined with any or all of Exercises 8-2, 8-3, and 8-5.

In This Exercise

You will bring a soil sample to class and check it for activity of ammonifying microbes that produce ammonia from organic nitrogen.

✓ Materials

Per Student

- ☐ One capped 25 mL tube of soil from a location of your choice (tubes should be handed out during the previous lab). This soil sample can be used for the other exercises relating to the nitrogen cycle (Exercises 8-2, 8-3, and 8-5).
- ☐ Two peptone water tubes (5 mL–8 mL)
- ☐ Two clear plastic microtubes
- ☐ Four transfer pipettes
- ☐ Two 15 × 100 test tubes
- ☐ Gloves

Per Student Group

- ☐ API Freshwater Master Test Kit[1]
  - Ammonia reagents #1 and #2
- ☐ Microfuge
- ☐ Digital balance and weigh boat
- ☐ Spatula
- ☐ (Optional) coarse screen

Medium Recipe

Peptone Water (Bartholomew)

• Peptone	40.0 g
• Distilled or deionized water	1.0 L

Procedure

Lab One

1. Obtain two peptone water tubes.
2. Try to remove as many rocks and sticks as possible from the soil. Passing it through a screen is helpful.
3. Weigh 1 gram of soil.
4. Transfer the 1 g of soil to one peptone water tube and label it "PW-Sample" or "PW" and the soil source. Leave the other uninoculated as a control and label it "PW-Control."
5. Incubate both PW tubes for up to 7 days at 25°C

Lab Two

1. Examine the inoculated tube for turbidity. You also may notice a distinctive odor. If there is none, you may wish to continue incubation for a few days longer.
2. Use a transfer pipette to dispense 1 mL of your peptone water culture into a clear plastic microtube. Close the cap tightly. Label it "PW-Sample."
3. Using a fresh transfer pipette, transfer 1 mL of uninoculated peptone water to the second clear plastic microtube. Label it "PW-Control 1."

[1] API Freshwater Test Kit is available from Mars Fishcare North America, Inc. PO Box 218, Chalfont, PA 18914, 1-800-847-0659 or www.aquariumpharm.com.

8

4 Label the two caps with your initials.

5 Spin both tubes for 2 minutes at high speed in a microfuge. Be sure to balance the microfuge by placing your tubes opposite from one another.

6 With a fresh transfer pipette, carefully draw 0.6 mL of broth out of your sample without disturbing the pellet at the bottom, and transfer to a clean glass test tube. Label this tube with your name and "PW-Sample."

7 With a fresh transfer pipette, carefully draw 0.6 mL of broth from the "PW-Control" and transfer to a clean glass test tube.

8 **Test for ammonia.** Use the reagents supplied to test for ammonia as follows:

- a Add one drop of ammonia reagent #1 and one drop of ammonia reagent #2 to "PW-Sample" and "PW-Control." Mix for 5 seconds. Hold the bottles upside-down, but vertically, in order to dispense the correct volume of reagent.
- b Watch for the color to develop in 5 minutes (Fig. 8.16A).
- c Compare the color against the color card to determine the ammonia concentration in your soil sample (Fig. 8.16B).
- d Record your results on the data sheet, page 607.

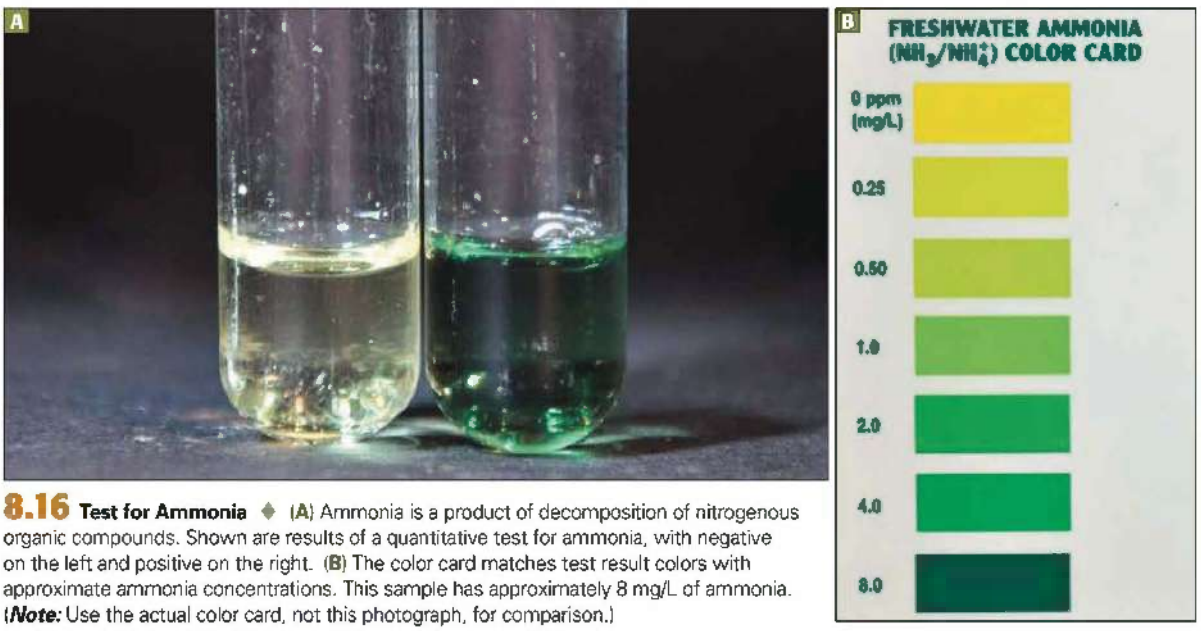
References

Atlas, Ronald and Richard Bartha. *Microbial Ecology—Fundamentals and Applications*, 4th ed. Menlo Park, CA: Addison Wesley Longman, 1998.

Bartholomew, James W. *Laboratory Textbook and Exercises in Microbiology*. Dubuque, IA: Wm. C. Brown Company Publishers, 1967.

White, David. *The Physiology and Biochemistry of Prokaryotes*, 2nd ed. New York and Oxford: Oxford University Press, 2000.

Willey, Joanne M., Linda M. Sherwood, and Christopher J. Woolverton. *Prescott's Principles of Microbiology*. Boston: McGraw-Hill Higher Education, 2009.



8.16 Test for Ammonia ◆ (**A**) Ammonia is a product of decomposition of nitrogenous organic compounds. Shown are results of a quantitative test for ammonia, with negative on the left and positive on the right. (**B**) The color card matches test result colors with approximate ammonia concentrations. This sample has approximately 8 mg/L of ammonia. (***Note:*** Use the actual color card, not this photograph, for comparison.)

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-4

Ammonification

OBSERVATIONS AND INTERPRETATIONS

Soil Sample Site: _____

1 Record your observations in the table below. Also record results from three classmates who worked with different soil.

Tube (include source of soil samples)	**Matching Color on Card**	**Ammonia Concentration (mg/L or ppm)**
Control		
Soil sample:		
Soil sample:		
Soil sample:		
Soil sample:		

QUESTIONS

1 *Consider the uninoculated tube.*

a. *Is it a positive or a negative control?*

b. *What information is provided by the uninoculated control?*

2 *If you did the other nitrogen cycle labs, why do you suppose you obtained such a strong reaction for the ammonification test, compared to the others?*

3 *If you did the other nitrogen cycle labs, what other process besides ammonification could be contributing to the results of this test?*

Denitrification: Nitrate Reduction

EXERCISE 8-5

Theory

Nitrate reduction is the result of anaerobic respiration, in which nitrate is used as the final electron acceptor. In some cases, nitrite is the end product, but in others nitrate is reduced to N_2 gas. We will be looking for the latter end product. Both processes are called **denitrification.** An example of this process is provided by *Pseudomonas denitrificans* and is shown below.

$$C_6H_{12}O_6 + 4NO_3 \rightarrow 6CO_2 + 6H_2O + 2N_2$$

Notice that this reaction is very similar to the summary reaction for aerobic respiration. Because the final electron acceptor is $4NO_3$ and not O_2, however, the products are different. O_2 is not required for—in fact, it often inhibits—anaerobic respiration, so dentrification usually occurs in its absence.

Because nitrate is so soluble, it is leached from soils into aquatic environments and ultimately resides in the oceans. Without denitrification, all the nitrogen would end up in the ocean and be unavailable to terrestrial life.

Application

This test allows for simple and fast determination of denitrification from soil samples. This exercise may be combined with any or all of Exercises 8-2, 8-3, and 8-4.

In This Exercise

You will bring a soil sample to class and check it for activity of denitrifying microbes that produce N_2 from nitrate.

✓ Materials

Per Student

- ☐ One capped 25 mL tube of soil from a location of your choice (tubes should be handed out the previous lab). This soil sample can be used for the other exercises relating to the nitrogen cycle (Exercises 8-2, 8-3, and 8-4).
- ☐ Two nitrate broths with Durham tubes
- ☐ Two clear plastic microtubes
- ☐ Two 15 × 100 test tubes
- ☐ Four transfer pipettes
- ☐ Gloves

Per Student Group

- ☐ API Freshwater Master Test Kit[1]
  - Nitrate reagents #1 and #2
- ☐ Microfuge
- ☐ Digital balance and weigh boat
- ☐ Spatula
- ☐ (Optional) coarse screen

Medium Recipe

Nitrate Broth

• Beef extract	3.0 g
• Peptone	5.0 g
• Potassium nitrate	1.0 g
• Distilled or deionized water	1.0 L

pH 6.8–7.2 at 25°C

Procedure

Lab One

1. Obtain two nitrate broth tubes.
2. Try to remove as many rocks and sticks as possible from the soil. Passing it through a screen is helpful.
3. Weigh 1 gram of soil.
4. Transfer the 1 g of soil to one nitrate broth tube and label it "NB-Sample" or "NB" and the soil source. Be sure the soil doesn't clog the opening of the Durham tube. Leave the other uninoculated as a control. Label it "NB-Control."
5. Incubate both NB tubes for up to 7 days at 25°C.

Lab Two

1. Examine the tube for turbidity.
2. Compare the Durham tubes of the sample and the control.
3. If you see a bubble in the sample tube and not in the control, proceed (Fig. 8.17). Otherwise, you may choose to let your culture incubate for a few more days.

[1] API Freshwater Test Kit is available from Mars Fishcare North America, Inc. PO Box 218, Chalfont, PA 18914. 1-800-847-0659 or www.aquariumpharm.com

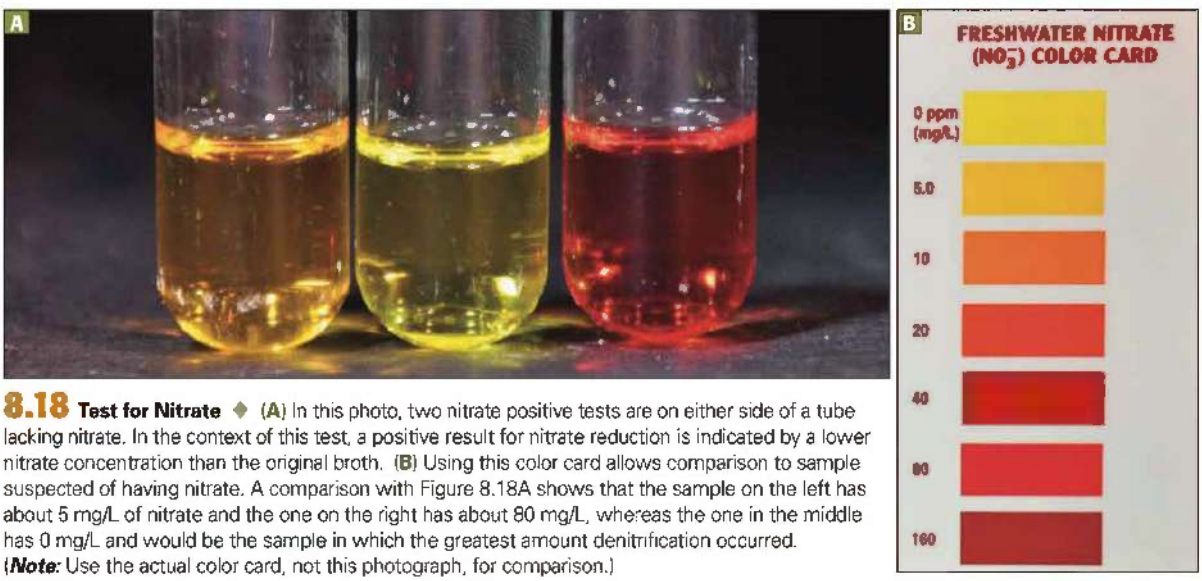
4. Transfer 1.5 mL from the "NB-Sample" tube to a clean, clear plastic microtube. Label it "NO_3-Sample." Label the cap with your initials.
5. Repeat for the "NB-Control" tube with a clean transfer pipette.
6. Spin both tubes in a microfuge for 2 minutes at high speed. Be sure to balance the microfuge by placing your tubes opposite one another.

8.17 Nitrate Reduction Broth with a Durham Tube ♦ The bubble in the Durham tube indicates that nitrate has been reduced to molecular nitrogen gas (N_2). This tube was inoculated with soil.

7. With a clean transfer pipette, carefully draw 1 mL of broth out of the "NO_3-Sample" tube without disturbing the pellet at the bottom and transfer it to a clean glass test tube. Label the tube with your name and "NO_3-Sample."
8. With a clean transfer pipette, transfer 1 mL from "NB-Control" to a clean glass test tube. Label it as "NB-Control."
9. **Test for nitrate.** Use the reagents supplied to perform the test for nitrate as follows:
   a. Holding the reagent bottle vertically, but upside-down, add two drops of nitrate reagent #1 to "NO_3-Sample" and to "NB-Control." Mix for 5 seconds.
   b. Shake nitrate reagent #2 for 30 seconds.
   c. Holding the bottle vertically, add two drops of nitrate reagent #2 to "NO_3-Sample" and to "NB-Control." Mix vigorously for 1 minute.
   d. Watch for color to develop in 5 minutes (Fig. 8.18A).
   e. Compare the color against the color card to determine the nitrate concentration in your soil sample (Fig. 8.18B).
   f. Record your results on the data sheet, page 611.

Reference

Atlas, Ronald and Richard Bartha. *Microbial Ecology—Fundamentals and Applications*, 4th ed. Menlo Park, CA: Addison Wesley Longman, 1998.



8.18 Test for Nitrate ♦ **(A)** In this photo, two nitrate positive tests are on either side of a tube lacking nitrate. In the context of this test, a positive result for nitrate reduction is indicated by a lower nitrate concentration than the original broth. **(B)** Using this color card allows comparison to sample suspected of having nitrate. A comparison with Figure 8.18A shows that the sample on the left has about 5 mg/L of nitrate and the one on the right has about 80 mg/L, whereas the one in the middle has 0 mg/L and would be the sample in which the greatest amount denitrification occurred. (***Note:*** Use the actual color card, not this photograph, for comparison.)

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-5

Denitrification: Nitrate Reduction

OBSERVATIONS AND INTERPRETATIONS

Soil Sample Site: _____

1 Record your observations in the table below. Also record results from three classmates who worked with different soil.

Tube (include source of soil samples)	Bubble in Durham Tube ("+" or "−")	Nitrate Concentration (mg/L or ppm)
Control		
Soil sample:		
Soil sample:		
Soil sample:		
Soil sample:		

QUESTIONS

1 *Consider the uninoculated nitrate broth tube.*

a. *Is it a positive or a negative control?*

b. *What information is provided by testing the uninoculated NB tube for nitrate?*

2 *If you performed other tests in the nitrogen cycle, you recall that you were told to look for turbidity in the broth before testing it. In this case, you were instructed to wait until there was a bubble in the Durham tube. Why isn't turbidity alone a good indicator that nitrate reduction has occurred?*

3 *How would the presence of a bubble in the Durham tube of the "NB-Control" affect your interpretation of results?*

4 *This test indicates nitrate reduction to N_2 gas by the accumulation of gas inside the Durham tube. This being the case, why do we perform a nitrate test with the reagents?*

5 *Suppose two nitrate broths are inoculated with 1 g of different soil samples. After incubation and testing one tube is yellow-orange and the other is brick red. In which tube would you expect the largest bubble and why?*

Sulfur Cycle—Introduction

Sulfur is one of the most abundant elements on Earth. Having oxidation states from −2 to +6, it is able to form many different compounds usable by living things. Most of the sulfur compounds used by microorganisms are inorganic molecules, used strictly for energy or to be incorporated into organic molecules in biosynthetic processes. Table 8-2 summarizes some important sulfur compounds and their oxidation states.

TABLE 8-2 Sulfur Compounds and How They are used

Sulfur Compound	Chemical Formula	Oxidation State	Used By Oxidizers	Used By Reducers
Organic sulfur	R–SH	−2	X	X
Sulfide	H_2S, HS^-, S^{2-}	−2	X	
Elemental sulfur	S^0	0	X	X
Thiosulfate	$S_2O_3^{2-}$	+2 per S	X	X
Sulfur dioxide	SO_2	+4		
Sulfite	SO_3^{2-}	+4		X
Sulfate	SO_4^{2-}	+6		X

The sulfur microorganisms are a diverse group and include both *Bacteria* and *Archaea*. They live in habitats as diverse as freshwater ponds, lakes, and rivers (especially where there is sewage contamination), water-saturated soils, saltwater lagoons, sulfur solfaturas as in Yellowstone National Park, and in and around deep ocean hydrothermal vents. This vast group includes photoautotrophs, photoheterotrophs, chemolithoautotrophs, chemolithoheterotrophs, obligate aerobes, facultative anaerobes, and obligate anaerobes.

Many sulfur oxidizers and reducers live **syntrophically** in mutually dependent communities, in which sulfur is converted back and forth between reduced and oxidized forms. Conversely, sulfur oxidizers, living in and around hydrothermal vents, although still a complex community, have a never-ending source of reduced sulfur flowing up from the vents. These microbes, receiving no biologically reduced sulfur, thrive in the ecosystem and produce large living mats that cover surrounding surfaces.

The exercises in this study unit focus on sulfur bacteria, all of which fall into three major categories—photoautotrophs, chemolithoautotrophs, and the sulfur reducers. Table 8-3 lists the major groups of sulfur bacteria and some summary reactions they perform.

The photoautotrophs are anoxygenic photosynthesizers, that is, they perform a type of photosynthesis that does not produce oxygen. These organisms reside in the anoxic zone of a pond or other aquatic ecosystem close enough to the surface to use the sun's energy to fix carbon from CO_2. Rather than chloroplasts, as in green plants, anoxygenic phototrophs contain membrane-bound bacteriochlorophyll. In sulfur bacteria, oxidation of H_2S or elemental sulfur provides electrons and bacteriochlorophyll traps light energy for ATP synthesis, both of which are used in carbon reduction and CO_2 fixation, respectively, in the production of carbohydrate. This reaction is analogous to the oxygenic photosynthetic reactions by cyanobacteria and eukaryotes.

photosynthetic eukaryotes: $CO_2 + H_2O \rightarrow [CH_2O] + O_2$

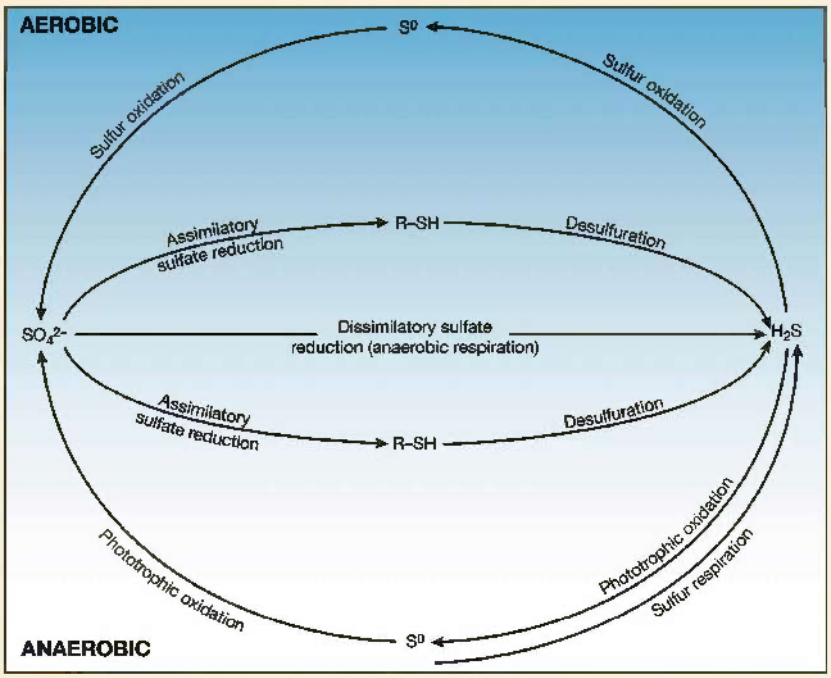
photosynthetic sulfur bacteria: $CO_2 + H_2S \rightarrow [CH_2O] + S^0$

TABLE 8-3 **Metabolic Reactions of Sulfur Bacteria**

Microbial groups	Representative Organisms	Habitat	Reactions	Summary Reactions
Photoautotrophs	*Chromatium*, *Chlorobium*	Anoxic	Anoxygenic photosynthesis	$H_2S + CO_2 \rightarrow S^0 + [CH_2O]$ $S^0 + CO_2 \rightarrow SO_4^{2-} + [CH_2O]$
Chemolithoautotrophs	*Beggiatoa*, *Macromonas*, *Thiobacillus*, *Thiobacterium*	Anoxic/oxic interface where H_2S and O_2 meet	Sulfur/sulfide/ thiosulfate oxidation	$HS^- + ½\ O_2 + H^+ \rightarrow S^0 + H_2O$ $H_2S + 2\ O_2 \rightarrow SO_4^{2-} + 2H^+$ $S^0 + 1½\ O_2 + H_2O \rightarrow H_2SO_4$ $S_2O_3^{2-} + H_2O + 2\ O_2 \rightarrow 2\ SO_4^{2-} + 2H^+$
Sulfate/sulfur reducers	*Desulfovibrio*, *Desulfobulbus*, *Desulfobacter*, *Desulfuromonas*	Either oxic or anoxic	Assimilatory sulfate reduction	$SO_4^{2-} \rightarrow S^{2-}$ + O-acetyl-L-serine $\rightarrow$ L-cysteine + acetate + H_2O
		Anoxic	Dissimilatory sulfate reduction	$SO_4^{2-} \rightarrow S^0$ $S^0 \rightarrow H_2S$
Many groups	Many organisms	Either oxic or anoxic	Desulfuration	Organic sulfur compounds $(R-SH) + H_2O \rightarrow R-OH + H_2S$

The chemolithoautotrophs are aerobic organisms that oxidize reduced sulfur compounds as an energy source and use it to fix carbon from CO_2. Because the most common form of reduced sulfur is sulfide gas (S^{2-}, HS^-, H_2S) produced in anoxic sediments, oxidation by these bacteria must occur as the gaseous sulfur rises and meets the oxic zone.

Sulfur reducers perform two important reduction reactions: dissimilatory and assimilatory sulfate reduction. Assimilatory sulfate reduction is the production of sulfide in the form of –SH groups needed for biosynthesis. Dissimilatory sulfate reduction is a purely energy releasing respiratory reaction. Finally, desulfuration (sulfur mineralization) is the reversal of assimilatory reduction and involves the release of H_2S to the environment. Figure 8.19 illustrates the major biogeochemical sulfur transformations. ◆



8.19 Biogeochemical Sulfur Transformations ◆ These are the major sulfur transformations in the sulfur cycle. Refer to Tables 8-2 and 8-3 for details.

EXERCISE 8-6

Photosynthetic Sulfur Bacteria

Theory

Both purple sulfur bacteria and green sulfur bacteria, although not closely related phylogenetically, appear in this exercise because they are both sulfur oxidizing anoxygenic phototrophs. In other words, they obtain energy from light, as do green plants and cyanobacteria, but without producing O_2. They reside in the anoxic regions of lakes, ponds, and marine environments that contain reduced sulfur compounds and sufficient light to drive their photosystems. Purple sulfur bacteria include members of *Chromatium*, *Thiopedia*, *Thiospirillum*, and *Ectothiorhodospira*. Green sulfur bacteria include members of *Chlorobium*, *Chlorobaculum*, and *Prosthecochloris*. Both purple and green genera contain bacteriochlorophyll, which absorbs light in the range of 350 nm–650 nm.

Purple and green sulfur bacteria each contain a photosystem that is similar to, but much simpler than, that of green plants. Green plants contain two photosystems to oxidize water (H_2O) molecules; photosynthetic sulfur bacteria each contain one photosystem to oxidize sulfide (H_2S). Although the former is oxygenic and the latter is anoxygenic, the systems are analogous.

All photosystems do one critical thing; they use light energy to convert a pigment molecule from a low-energy oxidant (accepts electrons) to a high-energy reductant (donates electrons). As the pigment molecule becomes energized by light and gains reduction potential, it passes electrons down an electron transport chain (ETC), thus releasing free energy, creating a proton motive force in the membrane, and producing ATP. For more information on redox reactions, see Exercise 5-1 Reduction Potential.

In the green sulfur bacteria photosystem (Fig. 8.20), the photo pigment P840 excites electrons (donated by H_2S or other reduced sulfur compounds to constituent cytochromes), which in turn are passed down an ETC to oxidized ferredoxin. The reduced ferredoxin then donates electrons to succinyl-CoA (a molecule in the citric acid cycle), which in turn combines with CO_2. With the help of ATP, the reduced succinyl-CoA eventually, through a series of redox reactions, is converted to citrate in what is called the **reverse citric acid cycle** (Fig. 8.20). Compare this reaction to the citric acid (Krebs) cycle in Appendix A.

The purple sulfur bacteria photosystem (Fig. 8.21) does roughly the same thing as the green sulfur bacteria photosystem with one important difference. In the purple sulfur bacteria photosystem, reduced coenzymes (NADPH) are required to transfer electrons to molecules in the Calvin cycle to complete the synthesis of carbon compounds.

However, the electrons needed to reduce $NADP^+$ must come from quinone—a molecule in the ETC with a lower reduction potential (Exercise 5-1) than that of the coenzyme. Therefore, the cell must spend ATP energy to transfer those electrons from quinone to $NADP^+$. This process is called **reverse electron flow** (Fig. 8.21).

The overall reaction of both green and purple sulfur bacteria could be summarized as follows:

$$CO_2 + H_2S \rightarrow [CH_2O] + S^0$$

Note: This is simply an overview of the major components involved. As explained earlier, the CO_2 and H_2S do not actually interact in this scenario. The elemental sulfur is produced as it donates electrons to the pigment molecule. CO_2, with the help of the newly reduced coenzymes, is converted to sugars either in the reverse citric acid cycle (green sulfur bacteria) or in the Calvin cycle (purple sulfur bacteria). The elemental sulfur produced by the purple sulfur bacteria is stored inside the cell. The elemental sulfur produced by green sulfur bacteria is stored outside the cell.

Application

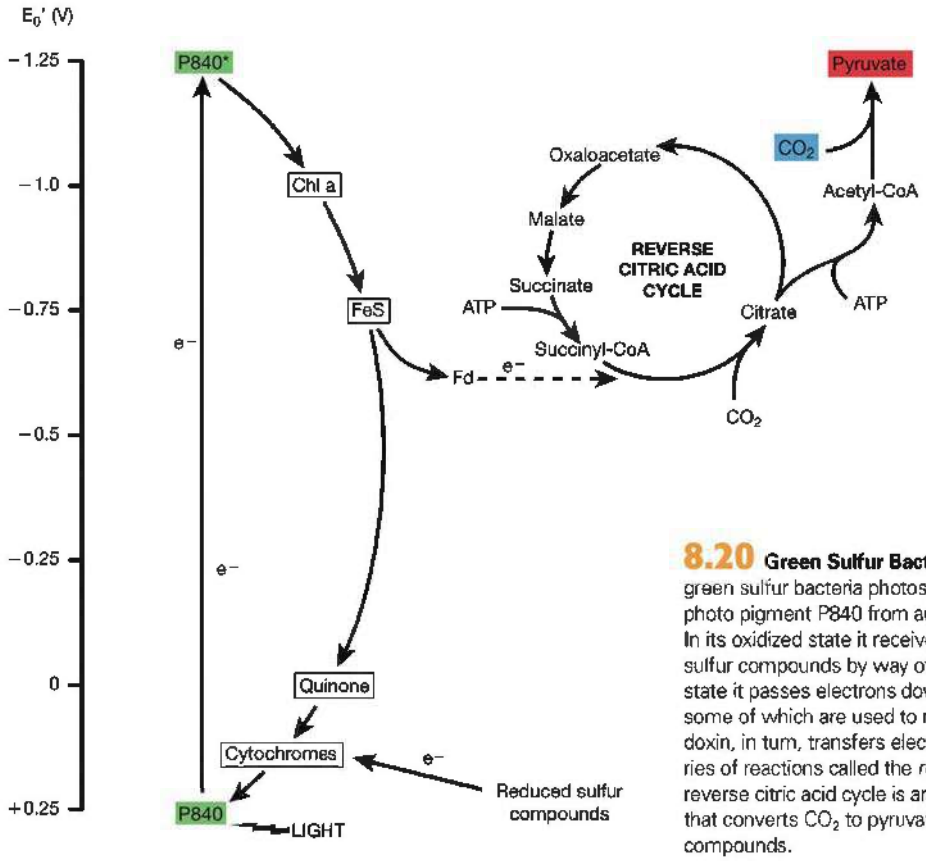
This exercise is for observation of phototrophic sulfur bacteria.

In This Exercise

The Winogradsky column (Fig. 8.1) illustrates some of the important growth regions for sulfur bacteria. Today, you will be harvesting bacteria from the column and observing them on the microscope. Refer to the figure and the photomicrographs of sulfur bacteria as you do the exercise.

✓ Materials

- ☐ Bunsen burner
- ☐ Pasteur or other glass pipette (long enough to reach the red and green zone of the Winogradsky column) with bulb
- ☐ Gloves
- ☐ Eye protection
- ☐ Forceps
- ☐ Microscope slides and coverslips
- ☐ Gram-stain materials

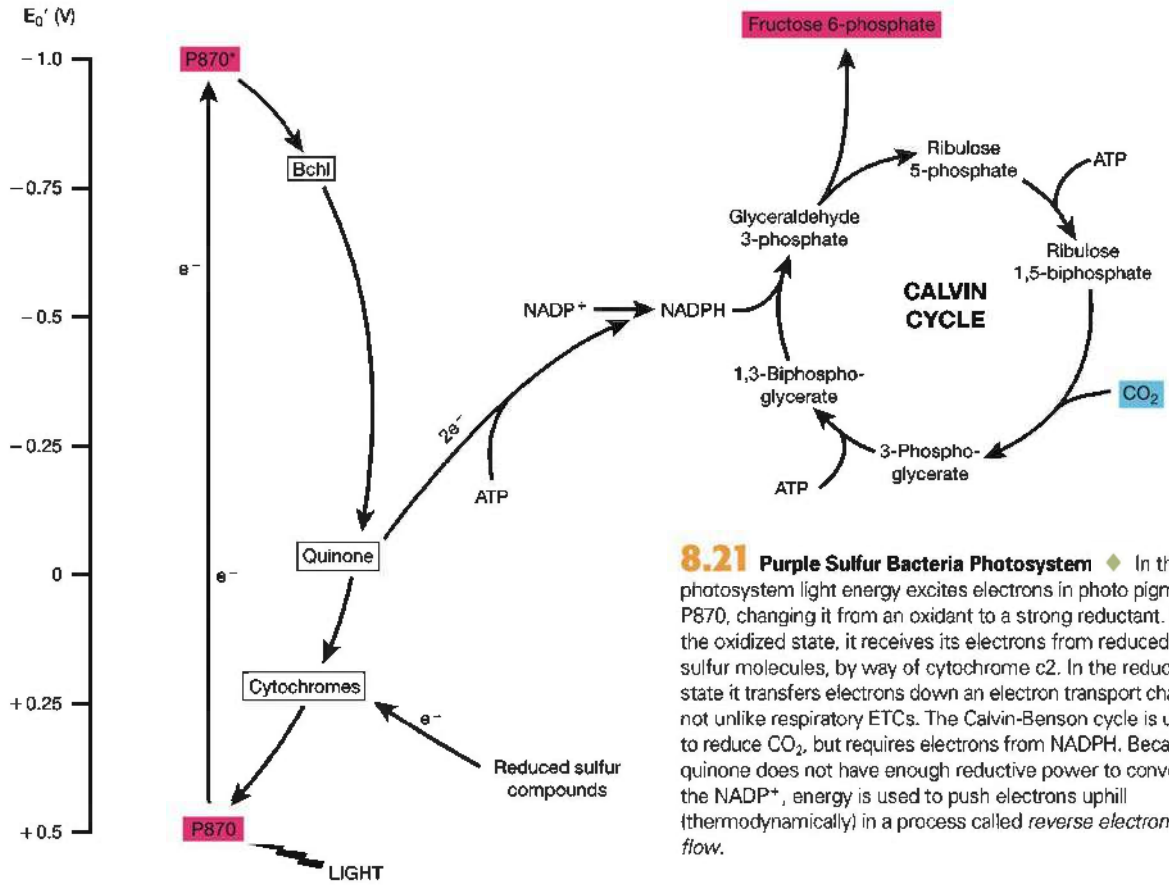


8.20 Green Sulfur Bacteria Photosystem ♦ In the green sulfur bacteria photosystem, light energy converts photo pigment P840 from an oxidant to a strong reductant. In its oxidized state it receives electrons from reduced sulfur compounds by way of cytochromes. In the reduced state it passes electrons down an electron transport chain, some of which are used to reduce ferredoxin (Fd). Ferredoxin, in turn, transfers electrons to succinyl-CoA in a series of reactions called the *reverse citric acid cycle*. The reverse citric acid cycle is an energy consuming reaction that converts CO_2 to pyruvate and other complex carbon compounds.

8

Procedure

1. Wearing eye protection and holding the glass pipette in one hand and the forceps in the other, heat the bottom ½–1 inch of the pipette in the Bunsen burner until it becomes soft. Using the forceps, gently bend the soft glass tip approximately 45–90 degrees (1/8–1/4 turn). Do not clamp down on the glass with the forceps and do not attempt to bend it too far. Doing either will close the tip and make the pipette unusable.
2. Referring to Figure 8.1, examine your Winogradsky column. To find your way to the correct organisms, let your eyes follow the various regions downward from the top. The layers you will observe are air, water, and a mud layer immediately below the water. Slightly below this layer is the beginning of the microaerophilic zone, which will be rust colored. This rust-colored zone will be inhabited by phototrophic, purple *non*-sulfur bacteria. Continue downward until you reach a red-violet region in the lower half of the column. This will be just above the green area very near the bottom of the column.
3. When you have found the red-violet area, tilt the column as far as you can without spilling the contents. Ease your bent pipette down the inside of the column until it reaches a spot with good color and remove a sample from next to the glass.
4. Place this sample on a microscope slide, add water as necessary, cover with a coverslip, and observe on the microscope. Watch for motility, and if you have a phase contrast scope, look for sulfur granules inside the cells. If you are doing a Gram stain, look for Gram-negative cells.
5. Repeat the above process for the green region. Here you are looking for green sulfur bacteria, also Gram negative, but with sulfur granules outside the cell.
6. Record your results on the data sheet, page 619, and answer the questions.



8.21 Purple Sulfur Bacteria Photosystem ◆ In this photosystem light energy excites electrons in photo pigment P870, changing it from an oxidant to a strong reductant. In the oxidized state, it receives its electrons from reduced sulfur molecules, by way of cytochrome c2. In the reduced state it transfers electrons down an electron transport chain, not unlike respiratory ETCs. The Calvin-Benson cycle is used to reduce CO_2, but requires electrons from NADPH. Because quinone does not have enough reductive power to convert the $NADP^+$, energy is used to push electrons uphill (thermodynamically) in a process called *reverse electron flow*.

References

Atlas, Ronald M. and Richard Bartha. Chap. 11 in *Microbial Ecology: Fundamentals and Applications*, 4th ed. Menlo Park, CA: Benjamin/Cummings Science Publishing, 1998.

Atlas, Ronald M. *Handbook of Microbiological Media*, 3rd ed. Boca Raton, FL: CRC Press, 2004.

Holt, John G., Noel R. Krieg, Peter H. A. Sneath, James T. Staley, and Stanley T. Williams. Groups 7, 10, and 12 in *Bergey's Manual of Determinative Bacteriology*, 9th ed. Baltimore: Lippincott Williams & Wilkins, 1994.

Madigan, Michael T. and John M. Martinko. Chaps. 12 and 17 in *Brock Biology of Microorganisms*, 11th ed. Upper Saddle River, NJ: Pearson Prentice Hall, 2006.

Maier, Raina M., Ian L. Pepper, and Charles P. Gerba. Chap. 14 in *Environmental Microbiology*, 2nd ed. Oxford, UK: Academic Press, 2009.

Radu Popa. Multiple personal interviews, June–October 2009.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-6

Photosynthetic Sulfur Bacteria

OBSERVATIONS AND INTERPRETATIONS

1 Sketch the cells you observed. Include the Gram reaction if applicable.

Sample #	Gram Stain Result	Sketch of Organisms Observed

QUESTIONS

1 *Why are the organisms looked for in this exercise found in the lower regions of the column?*

2 *In certain anoxic regions there are sulfur oxidizers and sulfur reducers living syntrophically. That is, each species gives the other species something that it needs. Which of the two phototrophs would you expect to see more of in such a habitat?*

3 *How is it possible for both green sulfur bacteria and purple sulfur bacteria to use ATP in the process of producing ATP without exhausting their energy stores?*

4 *Suppose that you could genetically engineer a strain of purple sulfur bacteria such that quinone (in the photosystem) was replaced by an electron carrier with a higher reduction potential than the redox pair* $NADP^+$*/NADPH. Upon cultivating them under identical conditions, what differences would you expect to see between the "wild" strain and the genetically engineered strain?*

Chemolithotrophic Sulfur-Oxidizing Bacteria

EXERCISE 8-7

Theory

Chemolithotrophic sulfur oxidizing bacteria are organisms that get their energy from reduced sulfur compounds. The most common electron donors for these organisms are hydrogen sulfide (H_2S), elemental sulfur (S^0), and thiosulfate ($S_2O_3^{2-}$). The term *chemolithotroph* distinguishes these organisms from photosynthetic sulfur bacteria (phototrophs) that also oxidize reduced sulfur compounds (Exercise 8-6).

Chemolithotrophic sulfur oxidizers live in aquatic habitats both in the water and in upper sediment layers. As such, they range from strictly aerobic to microaerophilic to facultatively anaerobic. Very common among the sulfur oxidizing bacteria are the so-called **gradient bacteria.** These microaerophiles require both oxygen and H_2S, but only in a very narrow range of concentrations. They typically reside at the level where H_2S rising from sediments and O_2 diffusing down from the air create a critical composition of the two gases.

Gradient bacteria, such as *Beggiatoa*, *Thioplaca*, and *Thiothrix* are facultative chemolithotrophs. They usually obtain their energy from H_2S and fix carbon from CO_2, but are capable of obtaining energy and carbon from organic compounds as well. The chemolithotrophic energy reaction is as follows:

$$H_2S + \tfrac{1}{2}\,O_2 \rightarrow S^0 + H_2O$$

These organisms typically store sulfur granules inside the cells (Fig. 8.22).

Thiobacillus is another group of sulfur oxidizing bacteria. Several species, such as *T. thioparus* and *T. novellus* perform the same reaction as the gradient bacteria and also store sulfur granules inside the cell. Other, more acidophilic *Thiobacillus* species such as *T. thiooxidans* and *T. ferroxidans*, oxidize sulfur compounds and elemental sulfur all the way to sulfuric acid as follows:

$$S^0 + 1\tfrac{1}{2}\,O_2 + H_2O \rightarrow H_2SO_4$$

Acidophilic *Thiobacillus* species are obligate chemolithotrophs and derive all of their energy and carbon from reduced sulfur compounds and carbon dioxide, respectively. Not surprisingly, they thrive in environments as acidic as pH 2.

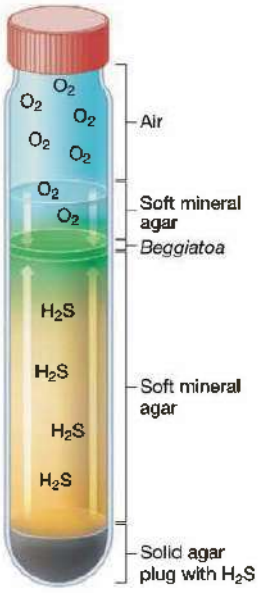
Most *Thiobacillus* species are obligate aerobes, but one species, *T. denitrificans*, is a facultative anaerobe important to the sulfur *and* the nitrogen cycle. As shown in the following reaction, it oxidizes elemental sulfur by reducing nitrate. This not only produces sulfate (SO_4^{2-}) needed by sulfur reducers, but converts nitrate to nitrogen gas.

8.22 Wet Mount of an Estuarine Sulfur Oxidizer (Bright Field) ◆ This is an unknown sulfur oxidizer isolated from estuarine mud. Note the clear spots indicating possible sulfur storage granules. (Depending on the plane of focus, these can look white, yellow, or blue.)

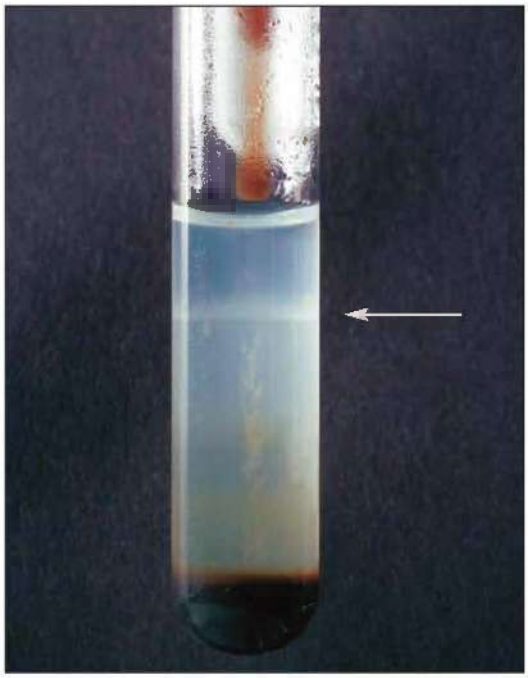
$$3S^0 + 4NO_3^- \rightarrow 3SO_4^{2-} + 2N_2$$

Two media were chosen for this exercise—a modified *Thiobacillus* medium and sulfur microgradient tubes. The *Thiobacillus* medium contains reduced sulfur and all of the necessary trace minerals and vitamins needed to isolate chemolithotrophic sulfur oxidizers. The broth is incubated on a shaker to maximize the oxygen content and encourage aerobic growth.

Microgradient tubes are made with two layers of agar—a soft layer on top and a solid layer on the bottom (Figs. 8.23 and 8.24). The bottom layer, called a "plug," contains concentrated hydrogen sulfide. The top layer is a soft mineral agar that provides necessary trace minerals and allows free movement of motile bacteria. The medium is first heated to remove the oxygen and then cooled to room temperature. Then it is stab inoculated and capped loosely. As the oxygen diffuses downward into the medium and the sulfide diffuses upward from the plug, the organism establishes a population in the area where optimal concentrations of both gases exist. This medium is best suited for gradient bacteria such as *Beggiatoa* and *Thiothrix*.



8.23 Microgradient Tube Diagram ♦ This is a diagram of a microgradient tube used to isolate *Beggiatoa*, a microaerophilic sulfur oxidizer. In the bottom of the tube is a solid agar "plug" containing sulfide (or other reduced sulfur compound). Above the plug is a deep soft mineral agar layer. The minerals provide necessary trace elements and help maintain the proper osmotic pressure and pH. Above the soft agar is air space. The sulfide from the plug diffuses upward through the soft agar and creates a gradient from high concentration at the bottom to low concentration somewhere above. The oxygen diffuses downward from the air space and creates its gradient from high concentration (~21%) at the top to low concentration below. The medium typically is stab inoculated with soil bacteria from a mixture of soil and water or buffer. The motile organisms move about freely and colonize the region that satisfies their needs.



8.24 Microgradient Tube ♦ This is a microgradient tube containing unknown soil bacteria. Microgradient tubes are commonly used to cultivate the sulfide oxidizer, *Beggiatoa*. Note the thin band of growth (arrow).

8

Application

This exercise is for observation of sulfur oxidizing bacteria.

In This Exercise

You will collect a sample of water and mud from a lake, pond, or other (indoor or outdoor) aquatic ecosystem and use your sample to cultivate aerobic and microaerophilic sulfur oxidizers in the media described. You will be given an opportunity to observe and to stain organisms that develop in the broth medium. The solid microgradient tubes are included for you to observe the very specific growth conditions required by gradient bacteria.

✓ Materials

Per Class

- ☐ Orbital shaker

Per Group

- ☐ Vortex mixer
- ☐ (Optional) pH paper (pH range 2–14)
- ☐ Pasteur pipettes with bulbs
- ☐ Microscope slides and coverslips
- ☐ Gram-stain materials
- ☐ Four tubes of modified *Thiobacillus* medium
- ☐ Three microgradient tubes
- ☐ Sediment/water sample from an aquatic ecosystem (in a clean glass container)

Medium recipe

Modified *Thiobacillus* Medium

(See Appendix H)

• Sodium thiosulfate	10.0 g
• K_2HPO_4	8.0 g
• KH_2PO_4	3.0 g
• NH_4Cl	0.4 g
• $MgSO_4 \cdot 7H_2O$	0.4 g
• Trace minerals solution (Appendix H)	2.0 mL
• Vitamin B_{12}	20.0 mg
• Biotin	10.0 mg
• Distilled or deionized H_2O	to 1.0 L

pH 7.5 adjusted with KOH

Microgradient Tubes

(Simplified version; see Appendix H.)

Top Layer

• Sodium thiosulfate	0.5 g
• Agar	2.5 g
• Trace mineral/salts solution	1.0 L

Bottom Layer (Plug)

• Na_2S	0.3 g
• Agar	15.0 g
• Trace mineral/salts solution	1.0 L

Procedure

Lab One

1. Obtain all materials for your group and label your tubes with your name, date, sample source, etc. Label the *Thiobacillus* broth tubes 1, 2, 3, and 4.
2. Shake your sediment sample well and transfer one drop to tube 1.
3. Vortex tube 1 well and, using a clean pipette, transfer one drop from it to tube 2.
4. Repeat this procedure with tubes 2 and 3. Do nothing to tube 4.
5. Using your inoculating loop, stab inoculate the microgradient tubes with a loopful of the (well-mixed) solution from tube 1. (*Note*: Stab the mineral agar portion only, and stop before you reach the sulfide agar plug in the bottom. Inoculate each tube individually, mixing well before each transfer.)
6. Place your four broths (along with all tubes from the class) in a rack on an orbital shaker set at medium speed in a 30°C incubator. Incubate for 24–48 hours, or longer if necessary to get turbidity.
7. Also incubate your microgradient tube at 30°C for 72 hours or until a thin line of microaerophilic growth appears as shown in Figure 8.24.

Lab Two

1. Remove your broths from the orbital shaker and examine them for turbidity.
2. Make wet mounts and Gram stains of organisms you find (Figs. 8.22 and 8.25).
3. Compare and share your slides with other students.
4. Using a clean pipette, place a drop of each broth on a separate strip of pH paper.
5. Record your results on the data sheet, page 625, and answer the questions.
6. Examine the gradient tubes for growth. Enter your observations on the data sheet.

8.25 Phase-Contrast Image of an Estuarine Sulfur Oxidizer ◆ This is a phase-contrast photomicrograph of unknown filamentous sulfur oxidizer cultivated from an upper layer of estuary sediment. The sulfur granules appear as white spots. Other white spots in the field may be sulfur granules in other sulfur oxidizing bacteria or they may be endospores. See Figure 8.1.

References

Atlas, Ronald M. and Richard Bartha. Chap. 11 in *Microbial Ecology: Fundamentals and Applications*, 4th ed. Menlo Park, CA: Benjamin/Cummings Science Publishing, 1998.

Atlas, Ronald M. *Handbook of Microbiological Media*, 3rd ed. Boca Raton, FL: CRC Press, 2004.

Holt, John G., Noel R. Krieg, Peter H. A. Sneath, James T. Staley, and Stanley T. Williams. Groups 7, 10, and 12 in *Bergey's Manual of Determinative Bacteriology*, 9th ed. Baltimore: Lippincott Williams & Wilkins, 1994.

Madigan, Michael T. and John M. Martinko. Chaps. 12 and 17 in *Brock Biology of Microorganisms*, 11th ed. Upper Saddle River, NJ: Pearson Prentice Hall, 2006.

Maier, Raina M., Ian L. Pepper, and Charles P. Gerba. Chap. 14 in *Environmental Microbiology*, 2nd ed. Oxford, UK: Academic Press, 2009.

Radu Popa. Multiple personal interviews, June–October 2009.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-7

Chemolithotrophic Sulfur-Oxidizing Bacteria

OBSERVATIONS AND INTERPRETATIONS

1 Sketch the cells you observed. Include the Gram reaction if applicable.

Modified *Thiobacillus* Broth			
Tube #	Gram Stain Result	pH	Sketch of Organisms Observed
1			
2			
3			
4			

Microgradient Tubes—Sketch Your Results		
1	2	3

QUESTIONS

1 *Why is the* Thiobacillus *medium shaken during incubation?*

2 *Many sulfur oxidizers can use organic carbon. In fact, enrichment media for several of these organisms include lactate or acetate. Why do you think there was no organic carbon added to the media used in this exercise?*

3 Beggiatoa *was discovered years before it could be cultivated in the laboratory. You likely cultivated it in this exercise. Why do you think it took so long to successfully cultivate it?*

EXERCISE 8-8

Sulfur-Reducing Bacteria

Theory

Sulfur-reducing bacteria perform two important types of sulfur reduction, **assimilatory sulfate reduction** and **dissimilatory sulfate reduction.** Assimilatory sulfate reduction is the process of actively transporting sulfate (SO_4^{2-}) into the cell and converting it to sulfhydryl groups (R–SH) found in the amino acids cysteine and methionine. Because sulfur is an essential component needed for biosynthesis, many bacteria, fungi, and even green plants transport it into their cells. Figure 8.26 illustrates a typical five-step conversion of sulfate to a sulfhydryl group in the synthesis of cysteine. The overall reaction can be summarized as follows:

$$SO_4^{2-} + ATP \rightarrow \text{cysteine} + H_2O.$$

Even though sulfide (S^{2-}) is the form needed by bacteria to generate sulfur-containing amino acids (reaction 5), sulfate (SO_4^{2-}) is the form actively transported and then converted inside the cell. This is because free sulfide reacts and precipitates with the iron in cytochromes,

$$H_2S + Fe^{2+} \rightarrow H_2 + FeS$$

thus, severely restricting electron flow and ATP production. The S^{2-} produced (in reaction 4) is instantly incorporated into a molecule of serine to stabilize it and complete the conversion to cysteine.

Dissimilatory sulfate reduction is a type of anaerobic respiration done exclusively by sulfate-reducing bacteria (SRB). Members of the SRB include *Desulfobacter, Desulfobulbus, Desulfococcus, Desulfotomaculum, Desulfonema, Desulfosarcina,* and *Desulfovibrio.* These organisms are part of an anaerobic bacterial consortium made up of fermenters, sulfate reducers, and methanogens that live in aquatic sediments. In the reaction, SO_4^{2-} is converted to H_2S, the reduced sulfur is immediately released back into the environment, and the freed electrons and energy are used to make ATP.

SRB are heterotrophs and must receive their carbon from organic molecules, but they can accept electrons from either inorganic or organic sources. Common electron donors for dissimilatory sulfate reduction are H_2 and a variety of low molecular weight organic molecules, such as acetate, alcohols, pyruvate, or lactate. Organic molecules are available in anaerobic habitats as products of fermentation and decomposition. Below are two common reactions. In each case, sulfide is released into the environment.

$$4H_2 + 2SO_4^{2-} \rightarrow S^{2-} + 4H_2O$$

$$4CH_3OH + 3SO_4^{2-} \rightarrow 4CO_2 + 3S^{2-} + 8H_2O$$

Some SRB can perform a **disproportionation reaction.** In this type of reaction, a sulfur compound (usually an intermediate such as thiosulfate, $S_2O_3^{2-}$ likely produced by a sulfur oxidizer) is split into two products, one of which is more oxidized than thiosulfate and one that is more reduced. A typical reaction would be:

$$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + H_2S$$

Note: The oxidation states are $S_2O_3^{2-} = +2$ (per S), $SO_4^{2-} = +6$, $H_2S = -2$. Therefore, the sulfate is more oxidized than thiosulfate and the hydrogen sulfide is more reduced.

8

1 SO_4^{2-} + ATP —*ATP sulfurylase*→ APS + PP_i

2 ATP + APS —*APS phosphokinase*→ PAPS + ADP

3 PAPS + thioredoxin_{red} —*PAPS reductase*→ SO_3^{2-} + PAP + thioredoxin_{ox}

4 SO_3^{2-} + 3NADPH —*NADPH–SO_3^{2-} reductase*→ S^{2-} + NADP

5 O-acetyl-L-serine + S^{2-} —*O-acetylserine sulfhydrylase*→ L-cysteine + acetate + H_2O

8.26 Assimilatory Sulfate Reduction ♦ This is an example of the multistep conversion of sulfate to the amino acid cysteine. Assimilatory sulfate reduction is performed by many bacteria. It solves the problem of obtaining an essential form of sulfur without dealing with the very toxic, free hydrogen sulfide. (***Note:*** One type of desulfuration, the release of sulfur from the cell, is the reverse of equation 5.)

Desulfuromonas acetoxidans is one of at least two species known to reduce sulfur rather than sulfate. In this example of anaerobic respiration the organism oxidizes acetate and converts the S^0 to H_2S as follows:

$$CH_3COOH + 2H_2O + 4S^0 \rightarrow 2CO_2 + 4H_2S$$

This reaction yields only a small amount of energy, but the organism survives for two important reasons: (1) it needs only the compounds shown in the formula for growth, and (2) it lives syntrophically in anoxic marine sediments with phototrophic green sulfur bacteria—*Chlorobium*. Each group provides an essential nutrient for the other. *Chlorobium* oxidizes the H_2S produced by *Desulfuromonas*; *Desulfuromonas* reduces the elemental sulfur released by *Chlorobium*. The acetate also comes from the green bacteria and other organics present in the environment.

Finally, there are a number of facultative anaerobes in the family *Enterobacteriaceae* that are able to reduce sulfur compounds. For more information on these organisms, see Exercises 5-20, 5-21, and 5-22.

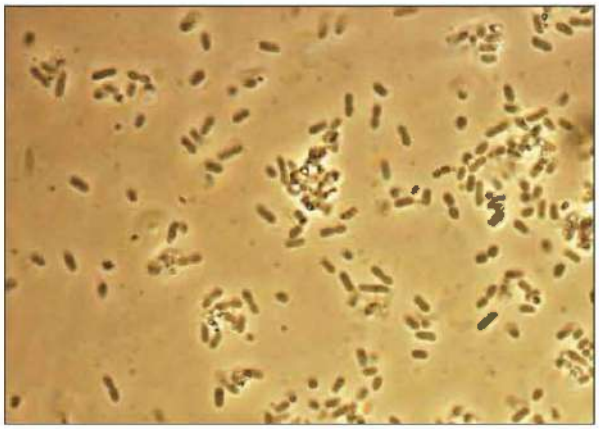
The medium chosen for this exercise, modified *Desulfovibrio* medium, contains lactate as an electron donor and both sulfite and sulfate (from the ferrous ammonium sulfate) as oxidized sulfur. Additionally, the ferrous ammonium sulfate serves as an indicator of sulfite or sulfate reduction by precipitating with any sulfide produced. Trace minerals and yeast extract take care of necessary growth factors while L-cysteine is added as a reducing agent to reduce oxygen to water and maintain an anoxic environment. The agar also slows oxygen diffusion but more importantly, prevents generalized turbidity that would occur if these motile organisms were allowed to move freely. Finally, ammonium phosphate and dipotassium phosphate act as buffers to maintain a relatively constant pH.

Application

This exercise is for observation of sulfate-reducing bacteria.

In This Exercise

You will collect a sample of sediment from a lake, pond, or other (indoor or outdoor) aquatic ecosystem and use your sample to cultivate sulfate-reducing bacteria (Figs. 8.27 and 8.28). In this lab you will perform a dilution series to attempt to cultivate individual colonies. Your instructor may or may not have you calculate the original concentration of SRB in the sample. The medium you will use—modified *Desulfovibrio* medium—is rich in sulfates and sulfites and encourages growth of organisms capable of reducing the oxidized sulfur.



8.27 Phase Contrast Image of a Sulfur Reducers ♦ This community of unknown sulfur reducers was recovered from black (anoxic) pond sediment. See Figure 8.1.

✓ Materials

Lab One

Per Class

- ☐ 50°C water bath

Per Student Group

- ☐ Vortex mixer
- ☐ Digital pipettes and sterile tips to transfer 1 mL of solution
- ☐ Six 9 mL dilution tubes
- ☐ Seven modified *Desulfovibrio* medium tubes (labeled 1–7) tightly closed (retained in the water bath until needed)
- ☐ Sediment sample from a pond, lake, or other aquatic ecosystem (in a clean glass container)
- ☐ Tube of filter-sterilized 0.5% ferrous ammonium sulfate

Lab Two

Per Student Group

- ☐ Microscope slides and coverslips
- ☐ Gram-stain materials

Medium recipe

Modified *Desulfovibrio* Medium

• DI H_2O	1.0 L
• Yeast extract	5.0 g
• Wolfe trace minerals (Appendix H)	10.0 mL
• Ammonium phosphate, dibasic	0.3 g
• Sodium lactate	3.0 g
• Sodium sulfite	2.0 g
• Dipotassium phosphate	1.7 g

8.28 *Desulfovibrio* Medium with Anaerobic Sulfur Reducers ♦ This medium can be used solely for cultivation or for quantitative purposes. It is heated immediately prior to inoculation to drive off the dissolved oxygen and melt the agar. The entire medium does not remain anoxic, but when inoculated and gently mixed, the organisms will populate the regions where oxygen has not returned by diffusion.

• L-Cysteine	0.5 g
• Ferrous ammonium sulfate	0.005 g
• Agar	3.0 g
pH 7.5 5 0.2 @ 25°C	

Procedure

Lab One

1. Obtain all materials for your group and label the dilution tubes 1–6. Set them aside temporarily.
2. Removing one *Desulfovibrio* tube from the water bath at a time (to prevent having it resolidify prematurely), aseptically add 1 mL 0.5% filter-sterilized ferrous ammonium sulfate to the medium, mix well, and return it to the water bath. Do this with all seven tubes. (It helps to use a digital pipette and placing the tip just below the surface of the medium, forcefully eject the iron sulfate, and then mix by rolling the tube between your hands. Use a different pipette for each transfer and do not shake the tube; this will introduce oxygen into the medium, which will inhibit growth of the desired organisms.
3. Start your dilution series (10^{-1} to 10^{-6}) by adding 1 g of sediment (1 mL if soupy) to dilution tube 1 (10^{-1}).
4. Vortex the tube very well and transfer 1 mL (while still homogeneous) to dilution tube 2 (10^{-2}).
5. Continue in this manner, transferring from tube 2 to 3, 3 to 4, etc., until all dilution tubes have received an inoculum.
6. Again, removing one medium tube from the water bath at a time, and using a different sterile pipette for each transfer, inoculate six medium tubes with 1 mL of its corresponding dilution. BE SURE TO VORTEX EACH *DILUTION* VERY WELL IMMEDIATELY BEFORE TRANSFERRING IT TO GROWTH MEDIUM. DO NOT VORTEX OR SHAKE THE MEDIUM. As before when adding the ferrous ammonium sulfate solution, place the pipette tip just below the top surface of the medium and eject the inoculum forcefully. Cap tightly, and mix well by rolling the tube between your hands. Do not inoculate tube 7.
7. Allow the medium tubes to solidify upright in a test tube rack, and then incubate them at 30°C for 48 hours to two weeks, checking daily for development of black colonies in the agar.

Lab Two

1. Remove your tubes from the incubator and examine them for black colonies in the agar.
2. Make wet mounts and Gram stains of organisms you find (Fig. 8.27). The organisms can be retrieved using an inoculating loop, needle, wooden applicator, or small spatula.
3. Compare and share your slides with other students.
4. Record your results on the data sheet, page 631, and answer the questions.
5. Be sure to dispose of all materials in appropriate autoclave containers.

References

Atlas, Ronald M. and Richard Bartha. Chap. 11 in *Microbial Ecology: Fundamentals and Applications*, 4th ed. Menlo Park, CA: Benjamin/Cummings Science Publishing, 1998.

Atlas, Ronald M. *Handbook of Microbiological Media*, 3rd ed. Boca Raton, FL: CRC Press, 2004.

Holt, John G., Noel R. Krieg, Peter H. A. Sneath, James T. Staley, and Stanley T. Williams. Groups 7, 10, and 12 in *Bergey's Manual of Determinative Bacteriology*, 9th ed. Baltimore: Lippincott Williams & Wilkins, 1994.

Madigan, Michael T. and John M. Martinko. Chaps. 12 and 17 in *Brock Biology of Microorganisms*, 11th ed. Upper Saddle River, NJ: Pearson Prentice Hall, 2006.

Maier, Raina M., Ian L. Pepper, and Charles P. Gerba. Chap. 14 in *Environmental Microbiology*, 2nd ed. Oxford, UK: Academic Press, 2009.

Radu Popa. Multiple personal interviews, June–October 2009.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-8

Sulfur-Reducing Bacteria

OBSERVATIONS AND INTERPRETATIONS

1 Enter your observations and data in the table. Include the Gram reaction if applicable. If you are calculating the original concentration of SRB in the sample, use the following formula:

$$OC = \frac{\text{colonies counted}}{\text{dilution}}$$

where OC = original concentration of SRB in cells/mL, colonies counted = the number of distinct colonies, and dilution = the dilution of the tube that was the inoculum source for the countable medium tube. (Refer to Exercise 6-1 for more information on dilutions and calculations.)

Tube #	Colonies Counted*	Gram Stain Result	Calculated SRB Concentration
1			
2			
3			
4			
5			
6			

* Enter the number of colonies if they are distinguishable from other colonies. If the tube is uncountable, enter "UC."

QUESTIONS

1 *What is the purpose of tube 7?*

2 *Assuming during incubation all tubes develop distinct black colonies, how could you verify whether or not your dilution technique was correct?*

3 *Which organism would you expect to have a higher metabolic rate (faster growth rate), a S^0 reducer or a SO_4^{2-} reducer? Why?*

4 *How would removing the iron from the formula, affect the number of colonies produced?*

EXERCISE
8-9

Bioluminescence

Theory

A few marine bacteria from genera *Vibrio* and *Photobacterium* are able to emit light by a process known as **bioluminescence**. Many of these organisms maintain mutualistic relationships with other marine life. For example, *Photobacterium* species living in the flashlight fish receive nutrients from the fish and in return provide a unique device for frightening would-be predators.

Bioluminescent bacteria are able to emit light because of an enzyme called **luciferase** (Fig. 8.29). In the presence of oxygen and a long-chain aldehyde, luciferase catalyzes the oxidation of reduced flavin mononucleotide ($FMNH_2$). In the process, outer electrons surrounding FMN become excited. Light is emitted when the electronically excited FMN returns to its ground state (Fig. 8.30).

It is estimated that a single *Vibrio* cell burns between 6,000 and 60,000 molecules of ATP per second emitting light. (ATP hydrolysis occurs in conjunction with synthesis of the aldehyde.) It also is known that their luminescence occurs only when a certain threshold population size is reached in a phenomenon called **quorum sensing**. This system is controlled by a genetically produced **autoinducer** that must be in sufficient concentration to trigger the reaction.

8.30 Bioluminescence on an Agar Plate ♦ This is an unknown bioluminescent bacterium growing on SWC Agar.

In This Exercise

You will inoculate seawater complete agar (SWC) with *Vibrio fischeri*, incubate it for a day or two, and then observe for bioluminescence.

✓ Materials

Per Student Group

- ☐ One seawater agar plate

Per Student

- ☐ One SWC plate

Medium Recipe

Seawater Complete Agar

• Peptone	5.0 g
• Yeast extract	5.0 g
• Beef extract	3.0 g
• Agar	15.0 g
• Seawater (synthetic)	1.0 L

8

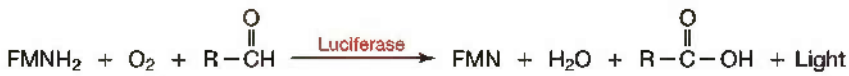
Procedure

Lab One

1. Obtain one SWC plate and aseptically inoculate it with *V. fischeri*. Any pattern will do.
2. Invert and incubate the plate at 25°C for 24 to 48 hours.

Lab Two

Remove the plate from the incubator and examine it in a dark room for light emission. It may take a while for your eyes to adjust to the dark and see the bioluminescence.



8.29 Chemistry of Bioluminescent Bacteria ♦ The enzyme luciferase catalyzes the oxidation of reduced flavin mononucleotide in the presence of an aldehyde. During the reaction, an electron becomes excited. When it returns to its ground state, light is emitted.

References

DIFCO Laboratories. *DIFCO Manual*, 10th ed. Detroit: DIFCO Laboratories, 1984.

Krieg, Noel R. and John G. Holt. Page 518 in *Bergey's Manual of Systematic Bacteriology*, Vol. 1. Baltimore: Lippincott Williams & Wilkins, 1984.

Power, David A. and Peggy J. McCuen. *Manual of BBL™ Products and Laboratory Procedures*, 6th ed. Cockeysville, MD: Becton Dickinson Microbiology Systems, 1988.

White, David. Pages 504–509 in *The Physiology and Biochemistry of Prokaryotes*. New York: Oxford University Press, 2000.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-9

Bioluminescence

QUESTIONS

1 *Considering the relationship between bioluminescent bacteria and the flashlight fish, what evolutionary advantage do you think quorum sensing gives the microorganism? Explain.*

2 *Why does the medium used for this exercise contain seawater?*

3 *If after 48-hours incubation, your organism has grown but does not emit light, what is the likely explanation? What should you do to correct the problem?*

8

EXERCISE 8-10

Soil Slide Culture

Theory

Trophic levels, food webs, symbioses, and species diversity and abundance are all important aspects of community structure studied by ecologists. However, physical location and proximity to other community members is also important. On a macroscopic level, this is relatively easy to assess. On the other hand, physical associations between soil microbes are difficult to ascertain without disturbing those relationships. That is, damage is done when one digs up the soil.

The soil slide culture is an easy way to see those relationships in situ. The idea is a simple one: a microscope slide is buried in soil and then carefully removed after a week or so. The microbes adhering to the slide are stained and examined under the microscope in their natural locations relative to one another and to soil particles.

This is a qualitative procedure in that community members and associations can be identified, and an idea of diversity can be assessed, but actual numbers and percentages cannot.

Application

The soil slide culture is used to view associations and diversity of soil microbes in situ.

In This Exercise

Your group will bring a soil sample to class, prepare it, and then bury microscope slides in it. After a week, you will observe the slides under the microscope and see what organisms are present and identify associations between each other and with soil particles.

✓ Materials

Per Student Group

- ☐ Soil sample (brought to class by a lab group member)
- ☐ One 600 mL (or other size depending on the size of each lab group) beaker
- ☐ One microscope slide per student
- ☐ Parafilm or plastic wrap and a rubber band
- ☐ Forceps (with tips capable of gripping a microscope slide by one end and lifting vertically)
- ☐ 40% v/v acetic acid
- ☐ Gloves
- ☐ Chemical eye protection
- ☐ Fume hood
- ☐ 1% rose bengal stain[1]
- ☐ Water bottle
- ☐ Beaker or other container in which to wash off the slides
- ☐ Two Coplin jars—one with the 40% acetic acid, the other with rose bengal stain
- ☐ Compound microscope

Stain Recipe

Rose Bengal Stain (1%)

• Rose bengal	0.1 g
• Distilled or deionized water	9.9 mL

Procedure

Lab One

1. Label the beaker with your lab group's name/number and soil type.
2. Add soil to the beaker until it is half full.
3. Add water to the soil so it is damp, but not saturated.
4. Each student should label one slide with his/her name, and then place it vertically in the soil with approximately 2 cm extending above the surface as in the preparation on the left in Figure 8.31. The label should be on the part above the soil!
5. Cover the beaker with Parafilm (or plastic wrap held on with a rubber band).
6. Poke a few holes in the Parafilm to allow aeration of the soil, but not so many that the soil will dehydrate during incubation. The number of holes will be determined by the size of the beaker used. The finished product should look like the preparation on the right in Figure 8.31.
7. Incubate for one week at room temperature.

[1] Available from Cole-Parmer, 625 East Bunker Court, Vernon Hills, IL 60061. http://www.coleparmer.com/Category/Rose_Bengal/37976?cls=37976.

8.31 Prepared Soil Cultures ♦ The beaker on the left shows three microscope slides properly buried in the moist, but not saturated, soil. The beaker on the right is covered with Parafilm and is ready for incubation.

8.32 Staining Set-up in a Fume Hood ♦ Shown are the Coplin jars of acetic acid and rose bengal stain on either side of a rinse pan. Notice that the slides being "fixed" in acetic acid have the labels up. Coplin jars are covered with Parafilm when not in use. Gloves and goggles must be worn while staining.

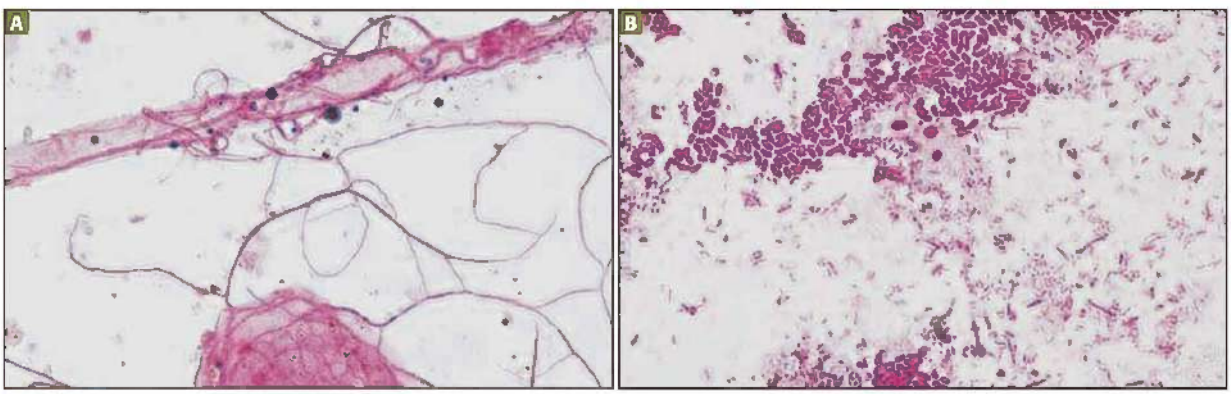
Lab Two

1. Remove the beaker from the incubator.
2. Remove your slide by tipping it *gently* to about 20 degrees so that the "upper" side is no longer in contact with the soil. This "upper" side is the one with your name. Then *gently* pull the slide out without rubbing or scraping the upper side.
3. *Gently* tap the slide on a paper towel on your bench top to remove chunks of soil. Thoroughly clean the lower surface with a damp paper towel. Let the slide dry at room temperature with the clean side down.
4. Your professor will have added 40% v/v acetic acid to one or more Coplin jars under a fume hood (Fig. 8.32). Wearing gloves and goggles use forceps to place your slide in one of the slots of a Coplin jar. Be sure to have your label at the top. Leave your slide in the acetic acid for 1 to 3 minutes.
5. Use forceps to remove your slide from the acetic acid. Then *gently* wash the acetic acid off your slide with water into the container provided.
6. Use forceps to place your slide in the rose bengal stain Coplin jar located under the fume hood. Leave the slide in the stain for 5 to 10 minutes.
7. Use forceps to remove your slide from the rose bengal stain and *gently* wash with water as in step 5.
8. Air-dry the slide on a paper towel at your desk.
9. Observe under oil immersion.
10. Compare your slide to Figures 8.33A and 8.33B. Record your results on the data sheet, page 639, and answer the questions.

References

Pepper, Ian and Charles Gerba. *Environmental Microbiology, A Laboratory Manual,* 2nd ed. Burlington, MA: Elsevier Academic Press, 2005.

Varnam, Alan H. and Malcom G. Evans. *Environmental Microbiology.* Washington, DC: ASM Press, 2000.



8.33 Micrographs of Soil Slide Cultures ♦ (A) The thick filament is a fungal hypha. The thin, branching filaments are streptomycetes. (B) Soil bacteria dominate this field. The plump rods in the cluster are probably *Azotobacter*. Both micrographs are ×1000.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-10

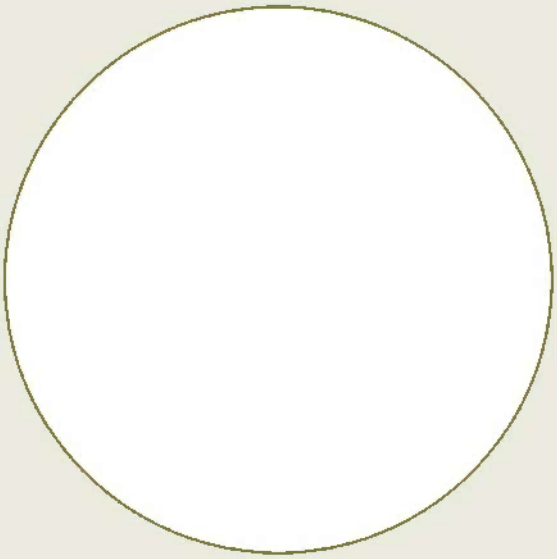
Soil Slide Culture

OBSERVATIONS AND INTERPRETATIONS

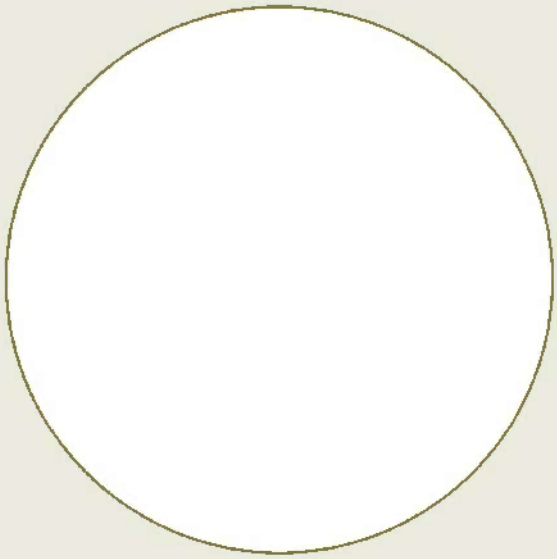
Soil Sample Site: _____

Length of incubation: _____

1 Observe two locations on your slide—one from near the surface and one from a deeper part of the soil—and sketch *relevant* parts of them in the circles below. "Relevant" means something showing a relationship of interest. That is, microbial associations with each other or with soil particles. Label any recognizable microbes.



Surface



Deep

QUESTIONS

1 *Why must the soil be kept moist but not too moist?*

2 *How can fungal hyphae be distinguished from streptomycetes?*

3 *Why might spore-forming bacteria be found in your slide communities?*

8

EXERCISE
8-11

Soil Microbial Count

Theory

Ordinary soil is a storehouse of microorganisms, both beneficial and pathogenic. Actinobacteria living in soil are important organic matter decomposers and humus producers. Some fungi are plant and animal pathogens, but most are saprophytes that decompose dead or dying plant material. Bacteria are by far the most numerous of the three groups. Some have animal reservoirs and are simply lying dormant as spores; others are important environmental nutrient cyclers (see Exercises 8-1 through 8-9). The major groups of antibiotic producing genera—*Bacillus*, *Cephalosporium*, *Penicillium*, and *Streptomyces*—can be found in soil.

The media used for this exercise are specifically designed for the organisms to be isolated. Glycerol yeast extract agar is designed for actinomycetes and does not offer enough nutritive value for typical bacteria or fungi. Nutrient agar is designed for a broad range of bacteria, but not fungi. Sabouraud dextrose agar is designed for fungi but also will support bacteria; therefore, penicillin and streptomycin have been added to discourage bacterial growth.

Application

This technique is used to determine the relative concentrations of bacteria, actinomycetes, and fungi in soil. It is also a good exercise to demonstrate diversity in soil samples.

In This Exercise

You will perform a serial dilution of a soil sample and calculate the densities of three very prominent soil residents—bacteria, actinomycetes, and fungi. Colony counts will be performed on plates produced using the pour plate technique (described in Exercise 6-5, Plaque Assay of Virus Titre). It is expected that the bacteria will outnumber the other groups and that the actinomycetes will outnumber the fungi. Therefore, the dilutions have been designed accordingly in order to produce countable plates for each group. To save time and material, the class will be divided into six groups. Refer to Table 8-4 for group assignments.

TABLE 8-4 Group Assignments

Group Number	Organism	Media (see materials)
1	Actinomycetes	GYEA
2	Actinomycetes	GYEA
3	Bacteria	NA
4	Bacteria	NA
5	Fungi	SDA
6	Fungi	SDA

✓ Materials

Per Student Group

- ☐ Soil sample
- ☐ Gloves
- ☐ Water bath set at 45°C containing sterile molten:
  - Sabouraud dextrose agar
  - Nutrient agar
  - Glycerol yeast extract agar
- ☐ Capped bottle containing 100 mL sterile water
- ☐ Micropipettes (10–100 µl and 100–1,000 µl) with sterile tips
- ☐ Sterile microtubes
- ☐ Flask of sterile water
- ☐ Five sterile Petri dishes
- ☐ Hand tally counter
- ☐ Colony counter

Medium Recipes

Sabouraud Dextrose Agar (with antibiotics added to inhibit bacteria)

• Peptone	10.0 g
• Dextrose	40.0 g
• Agar	15.0 g
• Penicillin	20,000.0 units
• Streptomycin	0.00004 g
• Distilled or deionized water	1.0 L
pH 5.4–5.8 at 25°C	

Nutrient Agar

• Beef extract	3.0 g
• Peptone	5.0 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L
pH 6.6 5 7.0 at 25°C	

Glycerol Yeast Extract Agar

• Glycerol	5.0 mL
• Yeast extract	2.0 g
• Dipotassium phosphate	1.0 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L

Procedure

Note: This is a quantitative exercise employing serial dilutions, dilutions, and calculations that are explained fully in Exercise 6-1, Standard Plate Count. If you have not completed a standard plate count, or you have any doubt about your ability to complete this exercise, we suggest that you read Exercise 6-1 carefully and do some or all of the practice problems.

Lab One: Groups 1 and 2

1. Obtain all materials, including 5 Petri dishes. Label the plates GYEA—A through E, respectively. Plate E will be the control.
2. Obtain 5 microtubes and label them 1 through 5. These are your dilution tubes; make sure they remain covered until needed.
3. Aseptically add 900 µl (0.9 mL) sterile water to dilution tubes 2, 3, 4, and 5. Leave tube 1 empty. Cover when finished.
4. Add 10 g of the soil sample to the 100 mL water bottle. Shake vigorously for several minutes. This is a 10^{-1} dilution. If you do not remember how to calculate dilutions, refer to Exercise 6-1.
5. Aseptically transfer 1,000 µl (1 mL) of the suspended solution from the bottle to dilution tube 1. Remember—this is 10^{-1}.
6. Aseptically transfer 100 µl from dilution tube 1 to dilution tube 2; mix well. This is 10^{-2}.
7. Aseptically transfer 100 µl from dilution tube 2 to dilution tube 3; mix well. This is 10^{-3}.
8. Aseptically transfer 100 µl from dilution tube 3 to dilution tube 4; mix well. This is 10^{-4}.
9. Tube 5 will remain sterile and be used as a control.
10. Remove 1 molten GYEA tube from the water bath. Aseptically add 100 µl from dilution tube 1, mix well, and pour into plate A. This plate contains 10^{-2} mL original sample volume. Repeat the process using 100 µl from tubes 2 through 5 to plates B through E, respectively. Label the plates with the original sample volume. Label plate E "control."
11. Allow the plates time to cool and solidify. Invert and incubate them at 25°C for 2 to 7 days.

Lab One: Groups 3 and 4

1. Obtain all materials, including 5 Petri dishes. Label the plates NA—A through E respectively. Plate E will be the control.
2. Obtain 5 microtubes and label them 1 through 5. These are your dilution tubes; make sure they remain covered until needed.
3. Aseptically add 900 µl (0.9 mL) sterile water to each dilution tube. Cover when finished.
4. Group 1 will have added 10 g of the soil sample to the 100 mL water bottle. (This is a 10^{-1} dilution. If you do not remember how to calculate dilution, refer to Exercise 6-1.)
5. Obtain the bottle from Group 1, mix the sample well, and aseptically transfer 100 µl of the suspended solution to dilution tube 1. Mix the contents of tube 1. This is 10^{-2}.
6. Aseptically transfer 100 µl from dilution tube 1 to dilution tube 2; mix well. This is 10^{-3}.
7. Aseptically transfer 100 µl from dilution tube 2 to dilution tube 3; mix well. This is 10^{-4}.
8. Aseptically transfer 100 µl from dilution tube 3 to dilution tube 4; mix well. This is 10^{-5}.
9. Tube 5 will remain sterile and be used as a control.
10. Remove 1 molten NA tube from the water bath. Aseptically add 100 µl from dilution tube 1, mix well, and pour into plate A. This plate contains 10^{-3} mL original sample volume. Repeat the process using 100 µl from tubes 2 through 5 to plates B through E, respectively. Label the plates with the original sample volume. Label plate E "control."
11. Allow the plates time to cool and solidify. Invert and incubate them at 25°C for 2 to 7 days.

Lab One: Groups 5 and 6

1. Obtain all materials, including 5 Petri dishes. Label five plates SDA—A through E, respectively. Plate E will be the control.
2. Obtain 4 microtubes and label them 1 through 4. These are your dilution tubes; make sure they remain covered until needed.

3. Aseptically add 900 µl (0.9 mL) sterile water to dilution tubes 2 through 4. Leave tube 1 empty. Cover when finished.
4. Group 1 will have added 10 g of the soil sample to the 100 mL water bottle. (This is a 10^{-1} dilution. If you do not remember how to calculate dilution, refer to Exercise 6-1.)
5. Obtain the bottle containing the sample, mix well and aseptically transfer 1,500 µl of the suspended solution to dilution tube 1. Remember—this is 10^{-1}.
6. Aseptically transfer 100 µl from dilution tube 1 to dilution tube 2; mix well. This is 10^{-2}.
7. Aseptically transfer 100 µl from dilution tube 2 to dilution tube 3; mix well. This is 10^{-3}.
8. Tube 4 will remain sterile and be used as a control.
9. Remove 1 molten SDA tube from the water bath. Aseptically add 1,000 µl (1.0 mL) from dilution tube 1; mix well and pour into plate A. This plate contains 10^{-1} mL original sample volume.
10. Remove another molten SDA tube from the water bath. Aseptically add 100 µl from tube 1 to an SDA tube; mix well and pour into plate B. Plate B contains 10^{-2} mL original sample volume. Repeat the process using 100 µl from tubes 2 through 4 to plates C through E, respectively. Label the plates with the original sample volume (the final dilution of plate A will remain the same as the source tube because it was inoculated with 1,000 µl rather than 100 µl). Label plate E "control."
11. Allow the plates time to cool and solidify. Invert and incubate them at 25°C for 2 to 7 days.

Lab Two: All Groups

1. After incubation, set aside the countable plates (those with 30 to 300 colonies) and properly dispose of the uncountable plates. Only one plate of each medium should be countable.
2. Count the colonies on the plate and calculate the original cell density (OCD) of the soil, using the formula provided.
3. Record the data on the chart in the data sheet, page 645.

References

Atlas, Ronald M., and Richard Bartha. Pages 367–374 in *Microbial Ecology—Fundamentals and Applications*, 4th ed. Menlo Park, CA: Benjamin/Cummings Science Publishing, 1998.

DIFCO Laboratories. Page 768 in *DIFCO Manual*, 10th ed. Detroit: DIFCO Laboratories, 1984.

Krieg, Noel R. and John G. Holt. Page 1383 in *Bergey's Manual of Systematic Bacteriology*, Vol. 1. Baltimore: Lippincott Williams and Wilkins, 1984.

Varnam, Alan H., and Malcolm G. Evans. Page 88 in *Environmental Microbiology*. Washington, DC: ASM Press, 2000.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-11

Soil Microbial Count

DATA AND CALCULATIONS

1 Count the colonies on the countable plates. Enter that number and the original sample volume in the table below. (*Note:* Remember that the "original sample volume" is the product of the volume and dilution of the liquid being plated (e.g., 0.1 mL × 10^{-2}).

Group	Colonies Counted	Original Sample Volume (mL)	Cell Density per mL of H_2O	Cell Density per Gram (g) of Soil
1				
2				
3				
4				
5				
6				

2 Calculate the number of "colony forming units" (CFUs) per mL of water for each group using the equation below. Record your answers in the table.

$$\text{Original CFU Density per mL of } H_2O = \frac{\text{Number of Colonies}}{\text{Original Sample Volume}}$$

3 Calculate the CFU density per gram of soil for each group using the equation below. Remember that the original sample had 10 g of soil in 100 mL H_2O. Record these values in the table.

$$\frac{\text{CFU}}{\text{g of Soil}} = \frac{\text{CFU}}{\text{mL } H_2O} \times \frac{100 \text{ mL } H_2O}{10 \text{ g of Soil}}$$

QUESTIONS

1 *What information was provided by the uninoculated plates (Plate E) of each medium?*

2 *Which type of organism was most abundant? Least abundant?*

3 *Considering only the dilution schemes for each type of organism, which would you predict to be the most abundant? Least abundant?*

4 *Calculations for this exercise could have been done in grams and milligrams because 1 milliliter of water weighs 1 gram, but the volume of water in the dilution bottle would have to have been adjusted to do this. To what should the volume be changed for calculations in grams to be correct?*

Water Quality Testing

Water quality is essential for maintaining public health. Appropriate community agencies routinely test drinking water and recreational bodies of water for microbial and chemical contamination. Obviously, it is the former with which we are concerned in the following exercises.

Many pathogens are transmitted through water contaminated with feces. Examples of bacterial pathogens include *Vibrio cholerae* (cholera), *Salmonella typhi* (typhoid fever), and *Shigella dysenteriae* (dysentery), among others. Protozoans, such as *Entamoeba histolytica* (amebiasis) and *Giardia lamblia* (giardiasis), and viruses (Hepatitis A) are also waterborne pathogens found in feces. It is not practical to screen water for every possible pathogen it might contain. Instead, water can be tested for fecal contamination using common intestinal flora as **indicator species**. For instance, *Escherichia coli* makes a good indicator species because it is abundant in feces, it is not fastidious and can survive with relative ease outside the body, and it is easily grown in the laboratory.

E. coli is a member of the *Enterobacteriaceae*, which is informally divided into **coliform** and **noncoliform** bacteria. The difference between the two groups is simply the ability of coliforms to ferment lactose to acid and gas end products. It is this ability that, within the context of the test system being used, identifies the presence of coliforms in general and sometimes *E. coli* in particular, both of which are indicative of fecal contamination.

Evidence of fecal contamination based on the presence of coliforms or *E. coli* does not mean the water is definitely carrying pathogens, or if it is, that the number of pathogens is likely to be high enough to cause infection. Standards have been established that set acceptable levels of coliform contamination in water, and these differ based on the intended use of it.

The following two exercises provide you with the opportunity to collect water samples and test them for fecal contamination. Because it is unknown if they are contaminated, or not, you will treat them as if they are, so please pay attention to the precautions in each exercise. ◆

EXERCISE 8-12

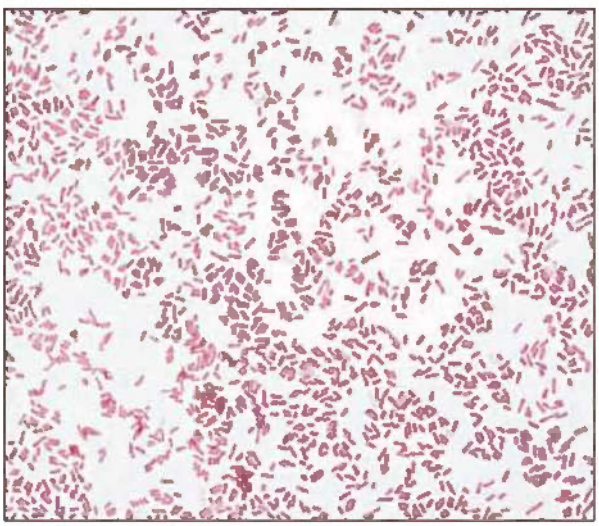
Membrane Filter Technique

8

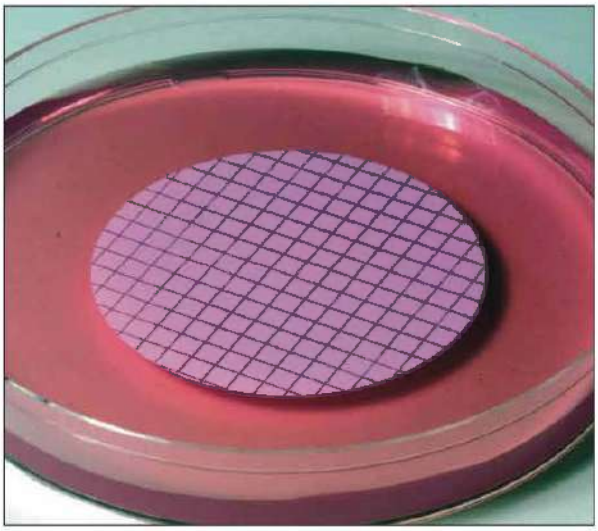
Theory

Fecal contamination is a common pollutant in open water and a potential source of serious disease-causing organisms. Certain members of *Enterobacteriaceae*, (Gram-negative facultative anaerobes) such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes*, are able to ferment lactose rapidly and produce large amounts of acid and gas. These organisms, called **coliforms**, are used as the indicator species when testing water for fecal contamination because they are relatively abundant in feces and easy to detect (Fig. 8.34). Once fecal contamination is confirmed by the presence of coliforms, any noncoliforms also present in the sample can be tested and identified as pathogenic or otherwise.

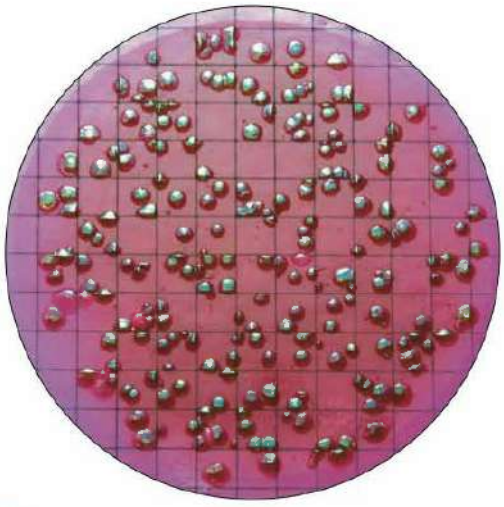
In the membrane filter technique, a water sample is drawn through a special porous membrane designed to trap microorganisms larger than 0.45 µm. After filtering the water sample, the membrane (filter) is applied to the surface of plated Endo agar and incubated for 24 hours (Fig. 8.35).



8.34 Gram stain of *Escherichia coli* ◆ *E. coli* is a principal coliform detected by the membrane filter technique. These cells were grown in culture.



8.35 Membrane Filter ◆ This porous membrane will allow water to pass through but will trap bacteria and particles larger than 0.45 µm. It is shown in position on an m-Endo agar LES plate.



8.36 Coliform Colonies on a Membrane Filter ◆ Note the characteristic dark colonies with gold metallic sheen, indicating that this water sample is contaminated with fecal coliforms. Potable water has less than 1 coliform per 100 mL of sample tested.

m-Endo agar LES is a selective medium that encourages Gram-negative bacterial growth and inhibits Gram-positive growth. It contains lactose for fermentation and basic fuchsin to indicate pH changes. Coliforms produce acetaldehyde, which causes colonies to appear red. Subsequent rapid lactose fermentation with acid end products causes the colonies to become mucoid with a metallic sheen. Noncoliform bacteria (including several dangerous pathogens) tend to be pale pink, colorless, or the color of the medium.

8

After incubation, all red or metallic colonies are counted and are used to calculate "coliform colonies/100 mL" using the following formula:

$$\frac{\text{Total coliforms}}{100\ \text{mL}} = \frac{\text{coliform colonies counted} \times 100}{\text{volume of original sample in mL}}$$

A "countable" plate contains between 20 and 80 coliform colonies with a total colony count no larger than 200 (Fig. 8.36). To assure that the number of colonies will fall within this range, it is customary to dilute heavily polluted samples, thereby reducing the number of cells collecting on the membrane. When dilution is necessary, it is important to record only the volume of *original sample* passed through the membrane, not any added water. Potable water contains less than 1 coliform per 100 milliliters of sample.

Application

The membrane filter technique is commonly used to identify the presence of fecal coliforms in water. While other media could be used, m-Endo agar LES is the standard in most applications.

In This Exercise

To determine total coliform population density, you will be using collected water samples and performing a membrane filter technique. Your results will be recorded in coliforms per 100 mL of sample.

✓ Materials

Per Class

- ☐ One broth culture of *Escherichia coli*
- ☐ One broth culture of *Staphylococcus epidermidis*
- ☐ Two sterile cotton swabs
- ☐ Sterile membrane filter suction apparatus (Fig. 8.37)
- ☐ Vacuum source (pump or aspirator)

Per Student Group

- ☐ One m-Endo agar LES plate
- ☐ One sterile membrane filter (pore size 0.45 µm)
- ☐ 100 mL water dilution bottle (to be distributed in the preceding lab)
- ☐ 100 mL water sample (obtained by student)
- ☐ 100 mL sterile water (for rinsing the apparatus)
- ☐ Gloves
- ☐ Household disinfectant and paper towels
- ☐ Hand sanitizer
- ☐ Small screw-cap jar containing alcohol and forceps
- ☐ Ziploc® bag large enough to hold the collection bottle

8.37 Membrane Filter Apparatus ◆ Assemble the membrane filter apparatus as shown in this photograph. Use two suction flasks (as shown) to avoid getting water into the vacuum source. Secure the flasks on the table, because the tubing may make them top-heavy.

Medium Recipe

m-Endo Agar LES

• Yeast extract	1.2 g
• Casitone	3.7 g
• Thiopeptone	3.7 g
• Tryptose	7.5 g
• Lactose	9.4 g
• Dipotassium phosphate	3.3 g
• Monopotassium phosphate	1.0 g
• Sodium chloride	3.7 g
• Sodium desoxycholate	0.1 g
• Sodium lauryl sulfate	0.05 g
• Sodium sulfite	1.6 g
• Basic fuchsin	0.8 g
• Agar	15.0 g
• Distilled or deionized water	1.0 L

pH 7.3–7.7 at 25°C

Procedure

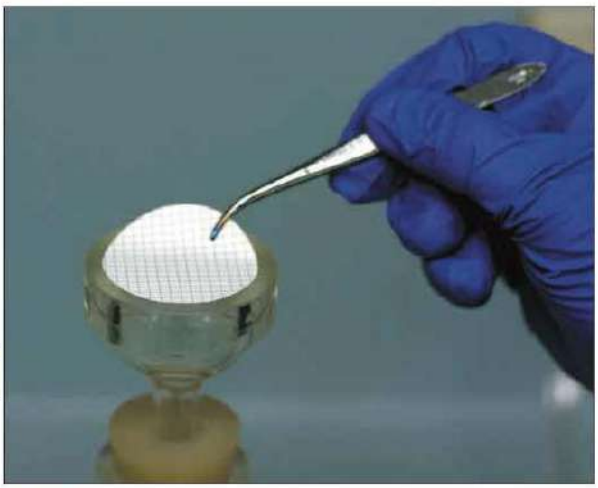
Prelab

1. Obtain a 100 mL water dilution bottle from your instructor. These have a white line inscribed on them that indicates the 100 mL level. (If you use another collection bottle or jar, it would be wise to mark the 100 mL level in lab with a permanent marking pen.)
2. Choose an environmental source to sample. You will be treating the site as potentially contaminated with feces. (Your instructor may decide to approve your choice to avoid duplication among lab groups.)
3. Visit the environmental site as close to your lab period as possible. Bring your water dilution bottle, a pair of gloves, some household disinfectant, hand sanitizer, and paper towels. While wearing gloves, fill the bottle to the white line (100 mL) and replace the cap.
4. Wipe the outside of the bottle with disinfectant. Dispose of the towels and gloves in the trash. Cleanse your hands thoroughly with hand sanitizer.
5. Put the bottle in the Ziploc® bag and close it.
6. Store the sample in the refrigerator until your lab period. If the sample must sit for a while before your lab, leave the cap loose to allow some aeration.

Lab One

1. Alcohol-flame the forceps (Fig. 8.38). Use care when flaming the forceps. Hold them away from the flame and the alcohol jar until the alcohol burns off of them. If the alcohol jar catches on fire, smother the flame with the jar's lid. Then, use the forceps to place the membrane filter (grid facing up) on the lower half of the filter housing (Fig. 8.39). Do not touch the filter with your fingers.
2. Clamp the two halves of the filter housing together and insert the filter housing into the suction flask as shown in Fig. 8.40. (This assembly can be a little top-heavy; so have someone hold it or otherwise secure it to prevent tipping.)
3. Pour the appropriate volume of water sample into the funnel. (Refer to Table 8-5 for suggested sample volumes. If the sample size is smaller than 10.0 mL, add 10 to 20 mL of sterile water before filtering, to help distribute the cells evenly on the surface of the membrane filter.)
4. Turn on the suction pump (or aspirator), and filter the sample into the flask.
5. Before removing the membrane, and with the suction pump still running, rinse the sides of the funnel two or three times with 20 mL or 30 mL of sterile water. This will wash off any cells adhering to the funnel and reduce the likelihood of contaminating the next sample.
6. Alcohol-flame the forceps again and carefully transfer the filter to the Endo agar plate by gently rolling it onto the surface grid side up. Be careful not to fold it or create air pockets (Fig. 8.41).
7. Wait a few minutes to allow the filter to adhere to the agar, then invert the plate and incubate it aerobically at 35 ± 2°C for 22 to 24 hours.
8. One group should spot inoculate an Endo agar plate with *E. coli* and *S. epidermidis*.

8.38 Alcohol-Flaming the Forceps ♦ Dip the forceps into the beaker of alcohol. Remove the forceps and quickly pass them through the Bunsen burner flame—just long enough to ignite the alcohol. **Be sure to keep the flame away from the jar of alcohol. If the jar catches fire, smother the flame with the jar's lid.**



8.39 Placing the Filter on the Filter Housing ♦ Carefully place the filter on the bottom half of the filter housing. Clamp the filter funnel over the filter.

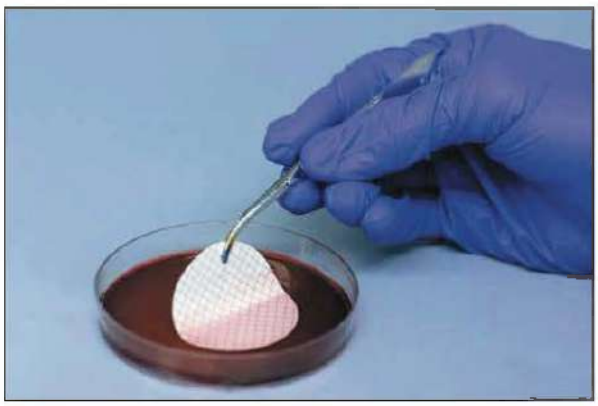
Lab Two

1. Remove the plate and count the colonies on the membrane filter that are dark red, purple, have a black center, or produce a green or gold metallic sheen.
2. Record your data in the table on the data sheet, page 653.
3. Calculate the coliform CFU per 100 milliliters using the following formula:

$$\frac{\text{Total coliforms}}{100\ \text{mL}} = \frac{\text{coliform colonies counted} \times 100}{\text{volume of original sample in mL}}$$

4. Record your results in the table on the data sheet.

8.40 Membrane Filter Assembly ♦ The membrane filter assembly is made up of a two-piece funnel and clamp. The membrane filter is inserted between the two funnel halves, and the whole assembly is clamped together.



8.41 Placing the Filter on the Agar Plate ♦ Using sterile forceps, carefully place the filter onto the agar surface with the grid facing up. Try not to allow any air pockets under the filter as you roll it onto the agar, because contact with the agar surface is essential for growth of bacteria. If air pockets form, try again. Allow a few minutes for the filter to adhere to the agar before inverting the plate.

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Page 270 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Eaton, Andrew D. (American Water Works Association), Lenore S. Clesceri (Water Environment Federation), Eugene W. Rice and Arnold E. Greenberg (American Public Health Association), and Mary Ann H. Franson. Chap. 9 in *Standard Methods for the Examination of Water and Wastewater*, 21st ed. Washington, DC: APHA Publication Office, 2005.

Zimbro, Mary Jo, and David A. Power, eds. Page 208 in *Difco™ and BBL™ Manual—Manual of Microbiological Culture Media*. Sparks, MD: Becton Dickinson and Co., 2003.

TABLE 8-5 **Suggested Sample Volumes for Membrane Filter Total Coliform Test**

Water Source	Volume (●) To Be Filtered (mL)							
	100	50	10	1.0	0.1	0.01	0.001	0.0001
Drinking water	●							
Swimming pools	●							
Wells, springs	●	●	●					
Lakes, reservoirs	●	●	●					
Water supply intake			●	●	●			
Bathing beaches			●	●	●			
River water				●	●	●	●	
Chlorinated sewage				●	●	●		
Raw sewage					●	●	●	●

(Reprinted with permission from the American Public Health Association)

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-12

Membrane Filter Technique

OBSERVATIONS AND INTERPRETATIONS

1 Enter the number of colonies counted in each group's sample and calculate the total coliforms per 100 milliliters of water using the following formula:

$$\frac{\text{Total coliforms}}{100\ \text{mL}} = \frac{\text{coliform colonies counted} \times 100\ \text{mL}}{\text{volume of original sample in mL}}$$

2 Enter the results in the table below.

Sample	Number of Colonies	Colonies/100 mL	Potable? Y/N

QUESTIONS

1 *Why is Endo agar used for this test instead of nutrient agar or tryptic soy agar? (The reason is the same for both.)*

2 *Consider the composition of m-Endo agar LES.*

a. *How would adding glucose to this medium affect the results?*

b. *Would glucose affect the medium's sensitivity or specificity? Explain.*

3 *Consider the Endo agar plate inoculated with* E. coli *and* S. epidermidis.

a. *What purpose did inoculating Endo agar with* E. coli *serve?*

b. *What purpose did inoculating Endo agar with* S. epidermidis *serve?*

4 *Suppose you were to count one coliform colony produced from a 10 mL water sample. What is the coliform density of the water in cells per 100 mL? Is the water potable? Explain.*

Multiple Tube Fermentation Method for Determining Water Quality

EXERCISE 8-13

Theory

The **multiple tube fermentation method**, also called **most probable number**, or **MPN**, is a common means of estimating the number of coliforms present in 100 mL of a sample using a statistical approach. It is not an actual counting of cells. The same procedure can be used to obtain the MPN for both **total coliforms** and *E. coli*.

The three media used in the procedure are: lauryl tryptose broth (LTB), brilliant green lactose bile (BGLB) broth, and EC (*E. coli*) broth. All three media contain lactose that coliforms ferment to acid and gas, the latter of which is used to indicate coliform presence. They also contain an ingredient that inhibits the growth of noncoliforms. In LTB the inhibitory agent is sodium lauryl sulfate. In BGLB and EC broth, the inhibitory agent is bile. Because LTB does not screen out all noncoliforms, it is used to *presumptively* determine the presence or absence of coliforms. BGLB broth is used to *confirm* the presence of coliforms because of its greater inhibitory effect on noncoliforms. EC broth, which includes lactose and bile salts, is selective for *E. coli* when incubated at 45.5°C.

All broths are prepared in 10 mL volumes and contain an inverted Durham tube to trap any gas produced by lactose fermentation. The LTB tubes are arranged in up to 10 groups of five (Fig. 8.42, Procedural Diagram). Each tube in the first set of five receives 1.0 mL of the original sample. Each tube in the second group receives 1.0 mL of a 10^{-1} dilution. Each tube in group three receives 1.0 mL of 10^{-2}, etc.[1] (*Note*: The volume of LT broth in the tubes is not part of the calculation of dilution factor. Dilutions of the water sample are made using sterile water prior to inoculating the broths. One milliliter of diluted sample is added to each tube in its designated group. Refer to Exercise 6-1 for help with serial dilutions.)

After inoculation, the LTB tubes are incubated at 35 ± 2°C for up to 48 hours, and then examined for gas production. (This is why the volume of LTB is not considered in the dilution. At this point, you are only concerned with the ability of a particular diluted water sample's ability to produce gas or not.). Any positive LTB tubes (Fig. 8.43) then are used to inoculate BGLB tubes. Each BGLB receives one or two loopfuls from its respective positive LTB tube (again, volumes are not critical). The cultures are incubated 48 hours at 35 ± 2°C and examined for gas production (Fig. 8.44). Positive BGLB cultures then are transferred to EC broth and incubated at 45.5°C for 48 hours (Fig. 8.45). After incubation the EC tubes with gas are counted. The same formula is used to calculate total coliform (BGLB) MPN and *E. coli* (EC) MPN, but they are calculated separately only using data from the appropriate tubes. The formula is:

$$\text{MPN/100 mL} = \frac{100P}{\sqrt{V_n V_a}}$$

Where

- P = total number of positive results (BGLB or EC)
- V_n = combined volume of sample in LTB tubes that produced negative results in BGLB or EC
- V_a = combined volume of sample in all LTB tubes

It is customary to calculate and report *both* total coliform and *E. coli* densities. Total coliform MPN is calculated using BGLB broth results, and *E. coli* MPN is based on EC broth results.

Using the data from Table 8-6 and the formula above, the calculation for total coliform MPN would be as follows:

$$\text{MPN/100 mL} = \frac{100P}{\sqrt{V_n V_a}}$$

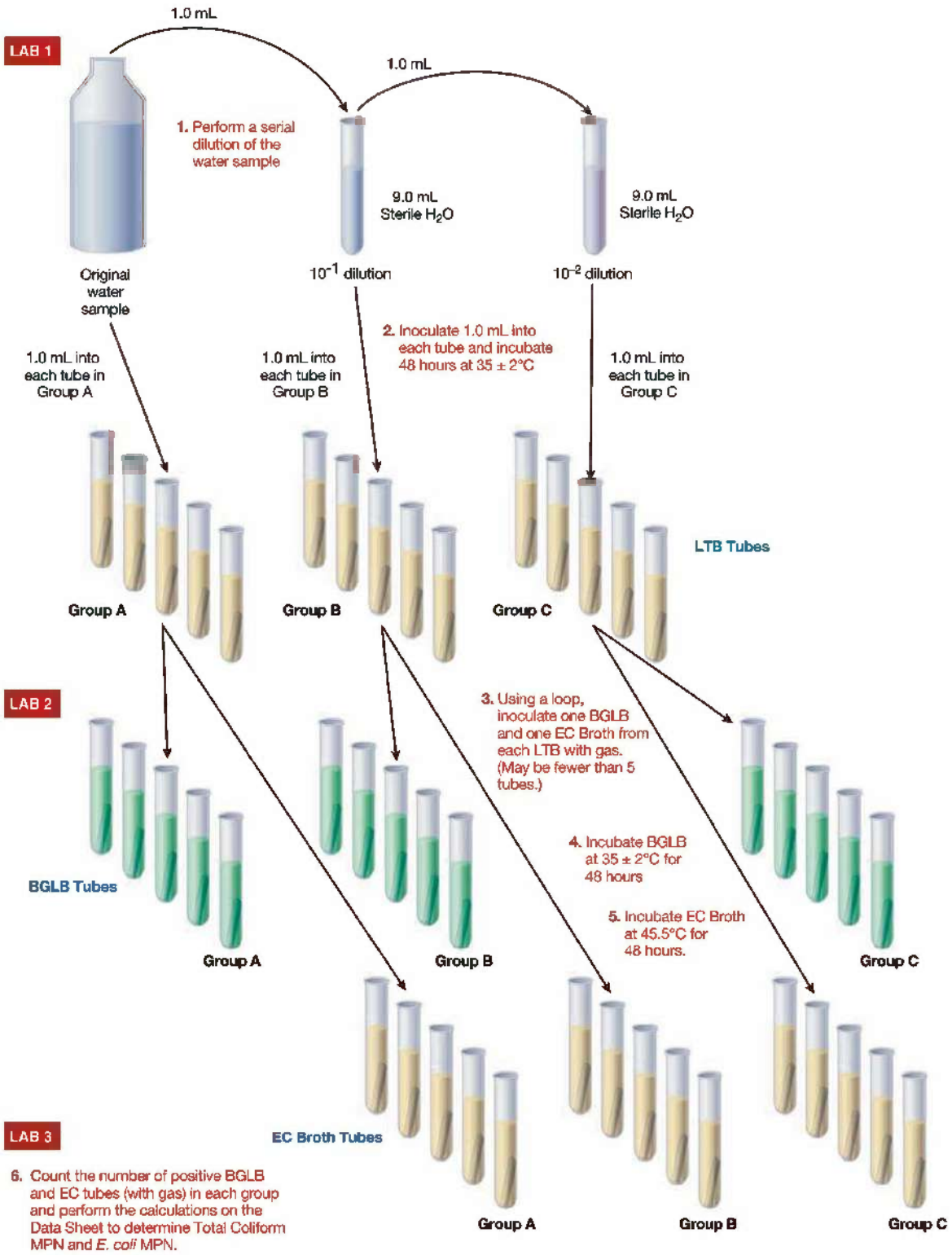
$$\text{MPN/100 mL} = \frac{100 \times 9}{\sqrt{0.24 \times 5.55}}$$

$$\text{MPN/100 mL} = 780$$

Application

This standardized test is used to measure coliform density (cells/100 mL) in water. It may be used to calculate the density of all coliforms present (total coliforms) or to calculate the density of *Escherichia coli* specifically.

[1] This exercise has been simplified for ease of instruction. The number of groups, number of tubes in each group, dilutions necessary, volume of broth in each tube, and volumes of sample transferred vary significantly, depending on the source and expected use of the water being tested. As a time-saver, we also have you inoculating BGLB and EC tubes from positive LTB, when the convention is to only inoculate EC tubes from positive BGLB tubes.


LAB 1
1.0 mL
1.0 mL
1. Perform a serial dilution of the water sample
9.0 mL Sterile H_2O
9.0 mL Sterile H_2O
Original water sample
10^{-1} dilution
10^{-2} dilution
2. Inoculate 1.0 mL into each tube and incubate 48 hours at 35 ± 2°C
1.0 mL into each tube in Group A
1.0 mL into each tube in Group B
1.0 mL into each tube in Group C
LTB Tubes
Group A
Group B
Group C
LAB 2
3. Using a loop, inoculate one BGLB and one EC Broth from each LTB with gas. (May be fewer than 5 tubes.)
4. Incubate BGLB at 35 ± 2°C for 48 hours
5. Incubate EC Broth at 45.5°C for 48 hours.
BGLB Tubes
Group A
Group B
Group C
LAB 3
EC Broth Tubes
6. Count the number of positive BGLB and EC tubes (with gas) in each group and perform the calculations on the Data Sheet to determine Total Coliform MPN and *E. coli* MPN.
Group A
Group B
Group C

8.42 Procedural Diagram of Multiple Tube Fermentation Method for Determination of Total Coliform ♦

8.43 LTB Results ♦ The bubble in the Durham tube on the right is *presumptive* evidence of coliform contamination. The tube on the left is negative.

8.44 BGLB Broth Results ♦ The bubble in the Durham tube on the right is seen as *confirmation* of coliform contamination. The tube on the left is negative.

8.45 EC Broth Results ♦ The bubble in the Durham tube on the right is seen as *confirmation* of *E. coli* contamination. The tube on the left is negative.

In This Exercise

You will be using collected water samples to perform a multiple tube fermentation. The data collected then will be used to calculate a total coliform count and an *E. coli* count. Counts will be recorded in coliforms per 100 mL of sample and *E. coli* per 100 mL of sample, respectively.

✓ Materials

Per Student Group

- ☐ 15 lauryl tryptose broth (LTB) tubes (containing 10 mL broth and an inverted Durham tube)
- ☐ Up to 15 brilliant green lactose bile (BGLB) broth tubes. (These tubes are needed for Lab Two. The number of tubes required will be determined by the results of the LTB test.)
- ☐ Up to 15 EC broth tubes. (These tubes are needed for Lab Two. The number of tubes required will be determined by the results of the LTB test.)
- ☐ Two sterile test tubes (minimum volume of 15 mL)
- ☐ Flask with 20 mL sterilized water
- ☐ One sterile 10.0 mL pipette
- ☐ A minimum of 5 sterile 1.0 mL pipettes
- ☐ Mechanical pipettor
- ☐ Water bath set at 45.5°C (Lab Two)
- ☐ Test tube rack
- ☐ Labeling tape
- ☐ 100 mL water dilution bottle (to be distributed in the preceding lab)
- ☐ 100 mL water sample (obtained by student)
- ☐ Gloves
- ☐ Household disinfectant and paper towels
- ☐ Hand sanitizer
- ☐ Ziploc® bag large enough to hold the collection bottle

Medium Recipes

Brilliant Green Lactose Bile Broth

• Peptone	10.0 g
• Lactose	10.0 g
• Oxgall	20.0 g
• Brilliant green dye	0.0133 g
• Distilled or deionized water	1.0 L
pH 7.0–7.4 at 25°C	

Lauryl Tryptose Broth

• Tryptose	20.0 g
• Lactose	5.0 g
• Dipotassium phosphate	2.75 g
• Monopotassium phosphate	2.75 g
• Sodium chloride	5.0 g
• Sodium lauryl sulfate	0.1 g
• Distilled or deionized water	1.0 L
pH 6.6–7.0 at 25°C	

EC Broth

• Tryptose	20.0 g
• Lactose	5.0 g
• Dipotassium phosphate	4.0 g
• Monopotassium phosphate	1.5 g
• Sodium chloride	5.0 g
• Distilled or deionized water	1.0 L
pH 6.7–7.1 at 25°C	

Procedure

Prelab

1. Obtain a 100 mL water dilution bottle from your instructor. These have a white line inscribed on them that indicates the 100 mL level. (If you use another collection bottle or jar, it would be wise to mark the 100 mL level in lab with a permanent marking pen.)
2. Choose an environmental source to sample. You will be treating the site as potentially contaminated with feces. (Your instructor may decide to approve your choice to avoid duplication among lab groups.)
3. Visit the environmental site as close to your lab period as possible. Bring your water dilution bottle, a pair of gloves, some household disinfectant, hand sanitizer, and paper towels. While wearing gloves, fill the bottle to the white line (100 mL) and replace the cap.
4. Wipe the outside of the bottle with disinfectant. Dispose of the towels and gloves in the trash. Cleanse your hands thoroughly with hand sanitizer.
5. Put the bottle in the Ziploc® bag and close it.
6. Store the sample in the refrigerator until your lab period. If the sample must sit for a while before your lab, leave the cap loose to allow some aeration.

Refer to the procedural diagram in Figure 8.42 as needed to complete the steps in Labs One and Two.

Lab One

1. Using the sterile pipette, dispense 9 mL of sterile water into each of the test tubes. These are the dilution blanks.
2. Mix the water sample. Then make a dilution by adding 1.0 mL of the water sample to one of the dilution blanks. Mix well. The 10 mL in this tube now consists of only 1/10 of the original sample; therefore, the dilution is 1/10 or 0.1 or 10^{-1} (preferred). Label the tube 10^{-1}. For help with dilutions, refer to Exercise 6-1.
3. Make a second dilution by adding 1.0 mL of the 10^{-1} dilution to the second dilution blank. Mix well. This 10 mL dilution contains 1/10 of 1/10 = 1/100 original sample and is 10^{-2}. Label the tube 10^{-2}.
4. Arrange the 15 LTB tubes into 3 groups of 5 in a test tube rack. Label the groups A, B, and C, respectively.
5. Mix the water sample once again. Then, aseptically transfer 1.0 mL of the undiluted water sample to each LTB tube in the group labeled A. You can use the same pipette for these transfers as long as you do not contaminate it. Mix well. This is undiluted sample; therefore it is a 10^{0} dilution.
6. Using a fresh, sterile pipette, aseptically add 1.0 mL of the 10^{-1} dilution to each of the LTB tubes in group B. Again, you may use the same pipette for each transfer as long as you do not contaminate it. Mix well. (***Remember:*** The volume of LTB in this step and step 7 is not part of the dilution calculation even though the original sample is more dilute.)
7. Using a fresh, sterile pipette, aseptically add 1.0 mL of the 10^{-2} dilution to each of the LTB tubes in the C group.
8. Incubate the LTB tubes at 35°C to 37°C for 48 hours.

Lab Two

1. Remove the LTB from the incubator and, one group at a time, examine the Durham tubes for accumulation of gas. Gas production is a positive result; absence of gas is negative if the broth is clear. If the LTB broth is turbid but contains no gas, the presence of coliforms is not yet ruled out. Therefore, record any turbidity as a positive result and include these tubes with those containing gas. Record positive and negative results in Table 1 on the data sheet, page 661, and calculate V_a.
2. Using an inoculating loop, inoculate one BGLB broth with each positive LTB tube. Make sure that each BGLB tube is *clearly labeled* A, B, or C according to the LTB tube from which it is inoculated.
3. Inoculate EC broths with the positive LTB tubes in the same manner as the BGLB above. Again, be sure to clearly label all EC tubes appropriately.
4. Incubate the BGLB at 35°C to 37°C for 48 hours. Incubate the EC tubes in the 45.5°C water bath for 48 hours.

Lab Three

1. Remove all tubes from the incubator and water bath and examine the Durham tubes for gas accumulation. At this point, turbidity does *not* count as positive; the only positive result is the presence of gas in the Durham tube. Count the positive BGLB tubes and enter your results in Table 2 on the data sheet, page 662.
2. Using Table 8-6 as a guide, complete Table 2 on the data sheet.
3. Using the data in Table 2, calculate the total coliform MPN using the formula on page 662. (This formula is used to calculate both Total Coliform MPN and *E. coli* MPN.)
4. Count the positive EC broth results in the same manner as the BGLB test. Record the results in Table 3 on the data sheet, page 662. Then, perform the calculations to complete Table 3 using the formula on page 662.
5. Determine the *E. coli* MPN in the same manner as described for total coliform count, and record your results in the chart on the data sheet.

Reference

Eaton, Andrew D. (American Water Works Association), Lenore S. Clesceri (Water Environment Federation), Eugene W. Rice and Arnold E. Greenberg (American Public Health Association), and Mary Ann H. Franson. Chap. 9 in *Standard Methods for the Examination of Water and Wastewater*, 21st ed. Washington, DC: APHA Publication Office, 2005.

TABLE 8-6 Example of BGLB Test Results ◆ The results shown here are of a hypothetical BGLB test using three groups of five tubes (A, B, and C). (***Note:*** On your data sheet, you are given three similar tables—one for LTB results, one for BGLB results, and one for EC broth results. Use this table as a sample to help you with completing Tables B and C using your own data.) In this example, the original water sample was diluted to 10^0, 10^{-1}, and 10^{-2}. The three dilutions were used to inoculate the broths in groups A, B, and C, respectively. **Row 1:** The dilution of the inoculum used per group. **Row 2:** The actual amount of original sample that went into each LTB tube. **Row 3:** The number of tubes in each group. **Row 4:** The number of tubes from each group of five that showed evidence of gas production. This total (in red) inserts into the equation as **P**. **Row 5:** The number of tubes from each group that did *not* show evidence of gas production. **Row 6:** Is used for calculating the "combined volume of sample in negative tubes" and refers to the inoculum that went into the LTB tubes that produced a negative result. This total (in red) inserts into the equation as $\mathbf{V_n}$. **Row 7:** Used for calculating the "combined volume of sample in all tubes" and refers to the total volume of inoculum that went into all LTB tubes. This total (in red) inserts into the equation as $\mathbf{V_s}$. As you can see, the undiluted inoculation (Group A) produced five positive results and zero negative results; the 10^{-1} dilution (Group B) produced three positive results and two negative; and the 10^{-2} dilution (Group C) produced one positive and four negative results. The total volume of original sample that went into LTB tubes was 5.55 mL, 0.24 mL of which produced no gas (shown in red in rows 7 and 6, respectively).

	Group	Group A	Group B	Group C	Totals (A + B + C)
1	Dilution (D)	10^0	10^{-1}	10^{-2}	NA
2	Volume of dilution added to each LTB tube that is original sample (1.0 mL × D)	1.0 mL	0.1 mL	0.01 mL	NA
3	# LTB tubes in group	5	5	5	NA
4	# BGLB positive results	5	3	1	9
5	# BGLB negative results	0	2	4	NA
6	Total volume of *original sample* in all LTB tubes that produced negative BGLB results (D × 1.0 mL × # negative tubes)	0 mL	0.2 mL	0.04 mL	0.24 mL
7	Volume of *original sample* in all LTB tubes inoculated (D × 1.0 mL × # tubes)	5.0 mL	0.5 mL	0.05 mL	5.55 mL

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET

8-13

Multiple Tube Fermentation Method for Determining Water Quality

DATA AND CALCULATIONS

1 Enter your LTB data here. Perform the calculations to complete the table. No MPN calculations are required.

Table 1 LTB Data—Presumptive Test

Group	Group A	Group B	Group C	Totals (A + B + C)
Dilution (D)	10^0	10^{-1}	10^{-2}	NA
Volume of dilution added to each LTB tube that is *original sample* (1.0 mL × D)	1.0 mL	0.1 mL	0.01 mL	NA
# LTB tubes in group	5	5	5	NA
# Positive results (Gas or turbidity)				P =
# Negative results (No gas or turbidity)				NA
Volume of *original sample* in all LTB tubes inoculated (D × 1.0 mL × # tubes)				V_a =

2 Enter your BGLB data here. Perform the calculations to complete the table.

Table 2 BGLB Data—Confirmation of Coliforms

Group	Group A	Group B	Group C	Totals (A + B + C)
Dilution (D)	10^0	10^{-1}	10^{-2}	NA
Volume of dilution added to each LTB tube that is *original sample* (1.0 mL × D)	1.0 mL	0.1 mL	0.01 mL	NA
# LTB tubes in group	5	5	5	NA
# BGLB positive results (Gas or turbidity)				P =
# BGLB negative results (No gas or turbidity)				NA
Total volume of *original sample* in all LTB tubes that produced negative results in BGLB tubes (D × 1.0 mL × # negative tubes)				V_n =
Volume of *original sample* in all LTB tubes inoculated (From Table 1)				V_a =

Calculate the total coliform MPN per 100 mL using the BGLB data.

Total coliform MPN/100mL $= \dfrac{100P}{\sqrt{V_n V_a}}$

3 Enter your EC broth data here. Perform the calculations to complete the table.

Table 3 EC Broth Data—Confirmation of *E. coli*

Group	Group A	Group B	Group C	Totals (A + B + C)
Dilution (D)	10^0	10^{-1}	10^{-2}	NA
Volume of dilution added to each LTB tube that is *original sample* (1.0 mL × D)	1.0 mL	0.1 mL	0.01 mL	NA
# LTB tubes in group	5	5	5	NA
# EC positive results (Gas)				P =
# EC negative results (No gas)				NA
Total volume of *original sample* in all LTB tubes that produced negative results in EC broth tubes (D × 1.0 mL × # tubes)				V_n =
Volume of *original sample* in all LTB tubes inoculated (from Table 1)				V_a =

Calculate the *E. coli* MPN per 100 mL using the EC broth data.

E. coli MPN/100mL $= \dfrac{100P}{\sqrt{V_n V_a}}$

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 8-13

(continued)

QUESTIONS

1 *In the following formula, V_n and V_a symbolize critical volumes in calculating the most probable number of coliforms in 100 milliliters of a sample. Do the symbols represent the amount of dilution added to tubes of LTB or the amount of original sample added to the tubes? Defend your answer in full and explain what would happen if you were to choose the wrong volumes in your calculation.*

$$\text{MPN/100 mL} = \frac{100\text{P}}{\sqrt{V_n V_a}}$$

2 *Suppose you were to run this test on a water sample and, after 48 hours incubation of the LTB tubes, found turbidity but no gas bubbles. What should you do next?*

3 *What if you found gas in the LTB tubes but none in the BGLB? How does this affect the EC test?*

4 *All coliforms ferment glucose, but none of the media used for this test includes glucose. Why do you think glucose is not used?*

5 *Would adding glucose increase or decrease the sensitivity? Specificity?*

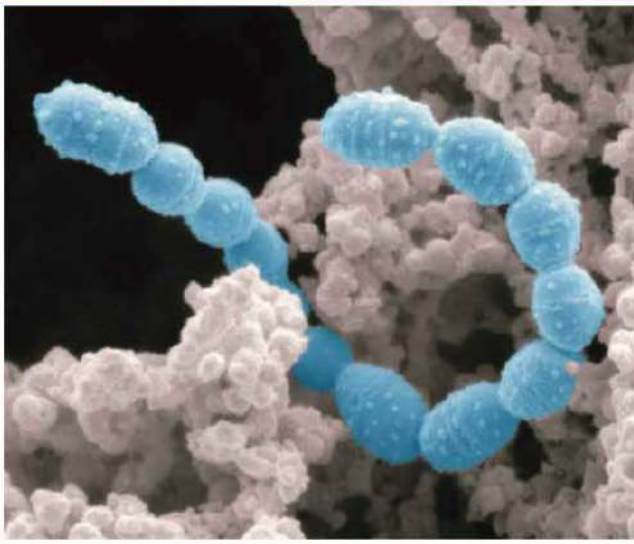
6 *Shown are data from row 4 of the BGLB and EC tables obtained after testing a hypothetical water sample.*

	Group	Group A	Group B	Group C	Totals (A + B + C)
1	Dilution (D)	10^0	10^0	10^0	NA
4	# positive BGLB results	5	3	2	10
4	# positive EC results	3	1	0	4

Explain the discrepancy in the number of positive tubes.

SECTION 9

Food Microbiology



Food microbiology is the study, utilization, and control of microorganisms in food. In Exercise 2-1 you learned that microorganisms exist virtually everywhere life exists. Left undisturbed in their own habitat, most are beneficial in some way. Some microorganisms have been used successfully to produce our favorite foods, such as yogurt, wine, beer, sauerkraut, buttermilk, vinegar, bread, and cheeses. Generally speaking, however, the unintended introduction of microorganisms to our food (including otherwise beneficial microorganisms) is a serious health hazard. For example, some strains of *Escherichia coli*, one of the most common enterics in humans and other mammals, can cause mild to severe illness or even death if ingested or introduced into other parts of the body.

It is not unusual to find unwanted microorganisms in unprocessed food. It is unavoidable and expected. This is why there are agencies such as the Food and Drug Administration (FDA) and the Centers for Disease Control and Prevention (CDC). These agencies were designed, in part, to protect consumers from the foodborne illnesses caused by improper management of food items. But these agencies exercise control only in the public arena; their influence over the practices of people in their homes typically is advisory only. This is why it is important to follow their recommendations and not only practice good personal hygiene, but also to wash and cook food properly.

In the first two exercises included in this section, you will be performing a simple test of milk quality and then you will have a little fun by making yogurt. Don't forget to wash your hands before you start! The last exercise combines a method (already introduced in Exercise 6-1) called the viable count with identification of an unknown. The sample will be a commercially produced yogurt product. You will estimate the number of colony forming units per gram of product and see if it meets minimum industry standards for labeling as "Live & Active Cultures." Then, you will isolate one species from the yogurt and identify it.

Methylene Blue Reductase Test

EXERCISE 9-1

Theory

Methylene blue dye is blue when oxidized and colorless when reduced. It can be reduced enzymatically either aerobically or anaerobically. In the aerobic electron transport system, methylene blue is reduced by cytochromes but immediately is returned to the oxidized state when it subsequently reduces oxygen. Anaerobically, the dye is in the reduced form, and in the absence of an oxidizing substance, remains colorless.

Milk is a good source of protein (casein) and carbohydrate (lactose), not only for mammals, but also for microbes—just think back at how many of the media used in earlier exercises had casein and/or lactose in them! Because it is such a good nutrient source and it has the potential of becoming contaminated with pathogenic microbes from the cow's skin or udder, or even unsterile machinery, milk quality is always a public health concern. The reduction of methylene blue may be used as an indicator of milk quality. In the methylene blue reductase test, a small quantity of a dilute methylene blue solution is added to a sterilized test tube containing raw milk. The tube then is sealed tightly and incubated in a 35°C water bath. The time it takes the milk to turn from blue to white (because of methylene blue reduction) is a qualitative indicator of the number of microorganisms living in the milk (Fig. 9.1). Good-quality milk takes longer than 6 hours to convert the methylene blue.

Application

This test is helpful in differentiating the *enterococci* from other streptococci. It also tests for the presence of coliforms in raw milk.

In This Exercise

You will test milk quality by measuring how long the indicator dye, methylene blue, takes to become reduced by any bacterial contaminants present.

9.1 Methylene Blue Reductase Test The tube on the left is a negative control to illustrate the original color of the oxidized medium. The tube on the right indicates bacterial reduction of methylene blue after 20 hours. The speed of reduction is related to the concentration of microorganisms present in the milk.

✓ Materials

Per Student Group

- ☐ Milk samples (raw or processed; a variety is best)
- ☐ Sterile screw-cap test tubes
- ☐ Sterile 1 mL and 10 mL pipettes with mechanical pipettors
- ☐ Hot-water bath set at 35°C
- ☐ Methylene blue reductase reagent
- ☐ Overnight broth culture of *Escherichia coli*
- ☐ Clock or wristwatch

Reagent

Methylene Blue Reductase Reagent

• Methylene blue dye	8.8 mg
• Distilled or deionized water	200.0 mL

Procedure

1. Obtain sterile tubes for as many samples as you are testing, plus two more, to be used as positive and negative controls. Label them appropriately.
2. Using a sterile 10 mL pipette, aseptically add 10 mL of milk to each tube.
3. Inoculate the tube marked "positive control" with 1 mL of *E. coli* culture.
4. Aseptically add 1.0 mL methylene blue solution to each test tube. Cap the tubes tightly and invert them several times to mix thoroughly.
5. Place the tube marked "negative control" in the refrigerator to prevent it from changing color.
6. Place all other tubes in the hot-water bath and note the time.
7. After 5 minutes, remove the tubes, invert them once to mix again, and then return them to the water bath. Record the time in the table in the data sheet, page 669, under "Starting Time" (t_S).
8. Using the control tubes for color comparison, check the tubes at 30-minute intervals, and record the time when each becomes white ("Completion Time"–t_C). Poor-quality milk takes less than 2 hours. Good-quality milk takes longer than 6 hours. If necessary, have someone check the tubes at 6 hours and record the results for you.
9. Using the table provided in the data sheet, calculate the "Elapsed Time" (t_E) it takes for each milk sample to become white.

References

Bailey, R. W. and E. G. Scott. Pages 114 and 306 in *Diagnostic Microbiology*, 2nd ed. St. Louis, MO: Mosby, 1966.

Benathen, Isaiah. Page 132 in *Microbiology with Health Care Applications*. Belmont, CA: Star Publishing, 1993.

Power, David A. and Peggy J. McCuen. Page 62 in *Manual of BBL™ Products and Laboratory Procedures*, 6th ed. Cockeysville, MD: Becton Dickinson Microbiology Systems, 1988.

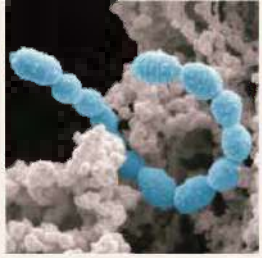
Richardson, Gary H., Ed. *Standard Methods for the Examination of Dairy Products*, 15th ed. Washington DC: American Public Health Association, 1985.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 9-1

Methylene Blue Reductase Test

OBSERVATIONS AND INTERPRETATIONS

1 Record your results and interpretations in the table below.

Milk Sample (brand and raw or processed)	**Starting Time (t_s)** (milk is blue)	**Completion Time (t_c)** (milk is white)	**Elapsed Time** ($t_E = t_C - t_S$)	**Milk Quality** (acceptable/ unacceptable)

QUESTIONS

1 *Why were you told to cap the tubes tightly? Explain.*

2 *What results would you expect if the tubes were inoculated with a strict aerobe? A strict anaerobe?*

EXERCISE
9-2

Making Yogurt

Theory

Several species of bacteria are used in the commercial production of yogurt. Most formulations include combinations of two or more species to synergistically enhance growth and to produce the optimum balance of flavor and acidity. One common pairing of organisms in commercial yogurt is that of *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophilus*.

Yogurt gets its unique flavor from acetaldehyde, diacetyl, and acetate produced from fermentation of the milk sugar lactose. The proportions of products, and ultimately the flavor, in the yogurt depend upon the types of enzyme systems possessed by the species used. Both species mentioned above contain constitutive β-galactosidase systems that break down lactose and convert the glucose to lactate, formate, and acetate via pyruvate in the glycolytic pathway. (See Appendix A.)

As you may remember, lactose is a disaccharide composed of glucose and galactose. *S. thermophilus* does not possess the enzymes needed to metabolize galactose, and *L. delbrueckii* preferentially metabolizes glucose. This results in an accumulation of galactose, which adds sweetness to the yogurt. Acetaldehyde is produced directly from pyruvate by *S. thermophilus* and through the conversion of proteolysis products threonine and glycine by *L. delbrueckii*. Some strains of *S. thermophilus* also produce glucose polymers, which give the yogurt a viscous consistency.

Application

This exercise is designed to keep you away from the Internet for a few minutes.

In This Exercise

You will produce yogurt using a simple home recipe with a commercial yogurt as a starter. Read the label to see which microorganisms are included. We hope you enjoy it.

✓ Materials

Per Student Group

- ☐ Whole, low-fat, or skim milk
- ☐ Plain yogurt with active cultures (bring from home or supermarket)
- ☐ Medium-size saucepan
- ☐ Medium-size bowl
- ☐ Wire whisk
- ☐ Hot plate
- ☐ Cooking thermometer
- ☐ Measuring cup
- ☐ Plastic wrap
- ☐ Fresh fruit
- ☐ (Optional) sugar
- ☐ pH meter or pH paper

Procedure

Lab One

1. Obtain all materials, and set them up in a clean work area.
2. While stirring, slowly heat 5 cups of milk in the saucepan to 185°F. Remove the milk from the heat, and let it cool to 110°F.
3. Place 1/4 cup starter yogurt in the bowl. Slowly, stir in cooled milk, about 1/3 cup to 1/2 cup at a time, mixing until smooth after each addition.
4. Cover the bowl with plastic wrap, and puncture several times to allow gases and excess moisture to escape.
5. Label the bowl with your name, the date, and the cultures present in your yogurt starter.
6. Incubate 5–6 hours at 30°C–35°C. Remove the bowl from the incubator at the correct time, and place it in the refrigerator.

Lab Two

1. Remove your yogurt from the refrigerator.
2. Perform a simple stain on a smear from your yogurt and examine with the microscope.
3. Compare flavor, consistency, and starter cultures with other groups in the lab. With a pH meter or pH paper, measure the pH of your yogurt. Record your results in the table provided on the data sheet, page 673.
4. Serve with fresh fruit and enjoy!

References

Downes, Frances Pouch and Keith Ito. Chapter 47 in *Compendium of Methods for the Microbiological Examination of Foods*, 4th ed. Washington, DC: American Public Health Association, 2001.

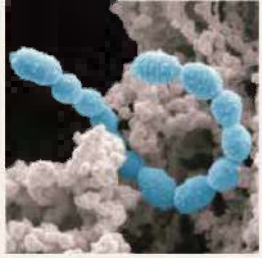
Ray, Bibek. Chapter 13 in *Fundamental Food Microbiology*, 2nd ed. Boca Raton, FL: CRC Press LLC, 2001.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 9-2

Making Yogurt

OBSERVATIONS AND INTERPRETATIONS

1 Record yogurt made by student groups below.

Culture Organisms	Cell Morphology and Arrangement	Flavor	Consistency	pH

Yogurt CFU Density of an Active and Live Culture and Identification of a Yogurt Unknown

EXERCISE 9-3

Theory

The National Yogurt Association (NYA) is a nonprofit organization whose members come from the yogurt industry. Their mission is to support "health and medical research for yogurt with live and active cultures and serve(s) as an information source to the trade and the general public"[1,2].

According to the standards set by the NYA, a yogurt manufacturer may use the "Live & Active Cultures" seal if their product has 10^8 CFU per gram (CFU/g) at the time of manufacture. (They actually say 10^8 "cultures," which is not correct, but they are writing for the general public, not microbiologists.)

The yogurt used in this lab will serve two purposes, neither of which has to do with eating it! First, you will perform a viable count to see if the chosen yogurt meets the minimum number of lactic acid bacteria to legitimately bear the seal of "Live & Active Cultures." The viable count is frequently used in quality control within the food industry, usually as a method to ensure that the degree of contamination is below a specified safe level. But in our application of it more microbes is better! Second, once you have done your quality control count on the yogurt, you will choose one of the isolated colonies to identify.

The idea behind the viable count is simple: At the minimum it takes only one cell to produce a colony on a plate (more on that later), so by counting the number of colonies on a plate the microbiologist can determine the number of cells that were inoculated onto it. For instance, if 65 colonies are counted after incubation, then the assumption is that the sample plated had 65 cells in it. Straightforward, right? Read on for the fine print.

First, we know that some bacteria grow as single cells, others as pairs or chains, and others in irregular clusters, so a colony may have actually come from more than a single cell[3]! In the example, it really isn't honest to report that the plate was inoculated with 65 cells because we don't know how many cells were stuck together in chains, pairs, or clusters to produce each colony. Therefore, we avoid overstating what we know by saying that the plate was inoculated with 65 *colony forming units*, or CFUs, rather than 65 cells.

A second factor to be added into the process is that rarely do we want to know the *total* number of microbes (or CFUs) in a sample. Usually we are more concerned with their *density*, that is, the number of microbes present in a given volume of sample. The convention is to report densities as CFU/mL. In the case of nonliquid samples, the standard units are CFU/g, and this is what the NYA uses (or means to)[4].

Lastly, you probably are wondering who (your lab partner?) is going to count 10^8 colonies on a Petri plate if the source was 1 g of yogurt. (Even if the sample came from 1 mg of yogurt, there would still be 10^5 colonies!) You couldn't even recover 10^8 colonies on a plate because they would grow together. We are confronted with two practical complications. Because the numbers we usually deal with are large: (1) they won't grow as individual colonies because the plate is too small and who wants to count that many colonies anyway, and (2) we like to work with plates that have between 30 and 300 colonies on them (some microbiologists use a 25–250 colony range). Why are these numbers chosen? One reason is to minimize the role of chance on the results: the fewer the colonies the greater its impact. For instance, if one plate has 30 colonies and another from the same sample has 29, an error of a little more than 3% has been introduced by chance, and that is unacceptable. The other reason for this range is that above 300 colonies we run into the

9

[1] AboutYogurt.com http://aboutyogurt.com/index.asp?bid=5, retrieved June 28, 2014.

[2] Just because they are nonprofit doesn't make them unbiased; nor can we assume that they are! Always maintain a healthy level of skepticism when reading information from *any* source! However, at the very least they *do* have an interest in promoting yogurt consumption by the public. The use of their information in this lab is restricted to their attempt to standardize and oversee ethical labeling of refrigerated and frozen yogurt products with respect to the phrase "Live & Active Cultures," so they are attempting to establish standards, which is something to be commended and not likely to be biased. Claims with respect to the health benefits of yogurt consumption, for instance, might need more scrutiny.

[3] Whether a CFU is one or a dozen cells doesn't affect the use of colonies for starting pure cultures. It only matters when trying to establish a density of microbes in a sample.

[4] This is necessary because a solid can have different volumes, so it can't easily and consistently be converted to mL. Frequently the solution to such conundrums is to establish conventions that admit to a certain degree of fudging, but it is always *consistent fudging*. The results obtained may not be accurate, but they are repeatable and comparable from one testing to another. Here, the convention is to use the fact that 1 mL of pure water at 25°C weighs 1 g. Simply apply the 1 mL equals 1 g, even though we know it is not true of yogurt.

crowding and the "too many to count" issues already mentioned. Notice, though, that the 30–300 (as well as the 25–250) range covers one power-of-ten increase. We like "tens" because they are easy to work with mathematically.

Therefore, instead of using 1 mL or 1 g in our testing, we can reduce the amount of original sample used. However, we are again limited by practicality: it is pretty hard to deliver 1 µL or weigh and deliver 1 µg of yogurt with accuracy and precision. Another approach to reducing the volume/mass of the original sample is to dilute it in small steps using manageable volumes (at this point, it is all about volumes even if the sample is a solid, because we start by adding the solid sample to a liquid). To keep the math simple, we usually do tenfold dilutions. Do this by taking 1 part of the sample and adding it to 9 parts of *diluent* (sterile saline or sterile water) to get a *dilution factor* of 10^{-1}. Or, take 1 part of sample and add it to 99 parts of diluent to get a dilution factor of 10^{-2}. We rarely do any dilutions other than these because we can link them together and perform a *serial dilution* (for an example, see Fig. 6.1). For instance, a 10^{-1} dilution of something that has already been diluted by a factor of 10^{-2} would produce a total dilution of 10^{-3}. (We could get a 10^{-3} by adding 1 part sample to 999 parts diluent, but at this point we would be using a very small volume of sample—1 µl in 999 µL—or a very large volume of diluent—1 mL in 999 mL. We frequently make dilutions of up to 10^{-8}, so the problem of one-step dilutions only worsens.)

In the second part of the lab, you will choose one colony and perform a series of tests to identify it. The yogurt sample analyzed must have a list of the microbes in it to use as a starting point, and most do. Then, the tests will provide results that you can compare to Tables 9-1 and 9-2 to get a provisional identification.

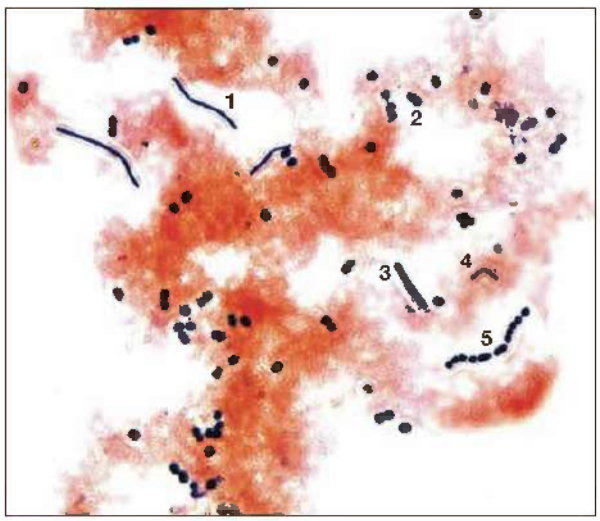
TABLE **9-1** **Structural Characters of Seven Bacterial Species Found in Yogurt**

Species	Cell Morphology	Cell Dimensions	Arrangement	Colony Morphology
Bifidobacterium spp. (Bifidus)	Irregular Rods		Single, may be branched	Smooth, convex, entire, cream to white, soft
Lactobacillus acidophilus	Long, rods; rounded ends	W 0.6–0.9 µm L 1.5–6 µm	Singles and chains	Rough, fuzzy outlines (TGA)
Lactobacillus casei	Average rods; squared ends	W 0.7–1.1 µm L 2.0–4.0 µm	Singles and chains	ND
Lactobacillus delbrueckii subsp. *bulgaricus*	Long, rods; rounded ends	W 0.5–0.8 µm L 2–9 µm	Singles and chains	ND
Lactobacillus. delbrueckii subsp. *lactis*	Long, rods; rounded ends	W 0.5–0.8 µm L 2–9 µm	Singles and chains	ND
Lactobacillus rhamnosus	Average rods; squared ends	W 0.8–1.0 µm L 2.0–4.0 µm	Singles and chains	ND
Streptococcus thermophilus	Ovoid cocci	D 0.7–1.0 µm	Pairs and chains	Small, round (~1 mm)

TABLE 9-2 **Physiological Characters of Seven Bacterial Species Found in Yogurt**

Species	Growth at 15°C	Growth at 45°C	VP	Acid from Galactose	Acid from Mannitol	Acid from Mannose	Acid from Salicin	Acid from Sucrose
Bifidobacterium spp. (Bifidus)	−	ND	−	−	−	−	+	+
Lactobacillus acidophilus	−	+	−	+	−	+	−	+
Lactobacillus casei	+	−	−	ND	+	ND	ND	+
Lactobacillus delbrueckii subsp. *bulgaricus*	−	+	−	−	−	−	−	−
Lactobacillus. delbrueckii subsp. *lactis*	−	+	−	d	−	+	+	+
Lactobacillus rhamnosus	+	+	−	ND	+	ND	ND	+
Streptococcus thermophilus	−	+	+	d	−	+	ND	+

+: ≥ 90% of strains are positive
−: ≥ 90% of strains are negative
d: 11–89% of strains are positive
ND: Not determined



9.2 Microbes in Yogurt ◆ This micrograph is a composite of several micrographs made from one Gram-stained specimen of commercial yogurt. There are at least four (or maybe five—it is not clear if #5 is a chain of #2 or another species) distinct cell morphologies.

Application

Viable counts are often used in the food industry as a means of quality control, either to assess the degree of microbial contamination of a product and ensure that it is safe to consume, or to verify that a minimum number of microbes is present as advertised.

In This Exercise

In today's lab you will be performing a viable count on a yogurt sample in order to determine if it meets the National Yogurt Association's standards for bearing the label "Live & Active Cultures." Then, you will choose one colony and perform a series of tests to identify it.

✓ Materials

Lab One

Per Class

- ☐ Commercially produced yogurt with a "Live & Active Cultures" seal and specific organisms listed in the ingredients
- ☐ Balance (must weigh at least 1 g)
- ☐ Weigh boat or paper
- ☐ 25 mL Erlenmeyer flask
- ☐ Spatula
- ☐ 10 mL graduated cylinder
- ☐ 9 mL sterile distilled water (sdH_2O)

Per Student Group

- ☐ Two 10–100 µL digital pipettors and a box of sterile tips
- ☐ Two 100–1,000 µL digital pipettors and a box of sterile tips
- ☐ One 25 mL flask of sterile dH_2O
- ☐ Six sterile 1.5 mL Eppendorf microtubes
- ☐ One Eppendorf microtube rack
- ☐ Six lactobacilli MRS agar plates
- ☐ Two glass spreading rods
- ☐ One beaker of alcohol
- ☐ Anaerobic jar with gas generator packet and indicator strip

Lab Two

Per Student Group

- ☐ Two hand counters
- ☐ Two Sharpie® markers or grease pencils
- ☐ Two lactobacilli MRS broth tubes
- ☐ Box of microscope slides and lens paper
- ☐ One TSB culture of *E. coli*

Lab Three

Per Student Group

- ☐ Two VP tubes
- ☐ Two BCP galactose tubes
- ☐ Two BCP mannitol tubes
- ☐ Two BCP mannose tubes
- ☐ Two BCP salicin tubes
- ☐ Two BCP sucrose tubes
- ☐ Four MRS broths
- ☐ Box of microscope slides and lens paper
- ☐ One TSB culture of *E. coli*
- ☐ 15°C water bath
- ☐ 45°C incubator or water bath

Lab Four

Per Student Group

- ☐ VP Reagents A and B
- ☐ Small test tube
- ☐ Nonsterile calibrated transfer pipette

■ Medium Recipes

Lactobacilli MRS Broth

• Proteose Peptone No. 3	10.0 g
• Beef extract	10.0 g
• Yeast extract	5.0 g
• Dextrose	20.0 g
• Polysorbate 80	1.0 g
• Ammonium citrate	2.0 g
• Sodium acetate	5.0 g
• Magnesium sulfate	0.1 g
• Manganese sulfate	0.05 g
• Dipotassium phosphate	2.0 g

Lactobacilli MRS Agar

- Same as MRS broth, but with 15.0 g agar.

Procedure

Lab One: Viable Count of Yogurt Culture

1. This first step will be done by the whole class.
   - a Mix the yogurt thoroughly.
   - b Weigh 1 g of mixed yogurt and transfer all of it to 9 mL of sterile dH_2O in a 25 mL Erlenmyer flask.
   - c Gently swirl the flask to thoroughly mix the diluted yogurt. This is a 10^{-1} dilution.
2. From here on, work with your lab partner.
   - a Gently swirl the flask of diluted yogurt and transfer 1,000 µL to a sterile microtube. Cap the tube and label it 10^{-1}.
   - b Using a sterile pipette tip, transfer 900 µL of sterile dH_2O to each of 5 sterile microtubes. Cap the tubes and label them 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Place them in order in the microtube rack along with the 10^{-1} microtube, leaving a space between each.
   - c With a sterile tip, use the digital pipettor to gently mix the contents of the 10^{-1} microtube by drawing up and dispensing the contents three or four times. Then, with a fresh tip immediately transfer 100 µl from the 10^{-1} microtube to the 10^{-2} microtube. Using the same tip, mix the contents of the 10^{-2} microtube.

d With a sterile tip, immediately transfer 100 µL from the 10^{-2} microtube to the 10^{-3} microtube and mix as before.

e With a sterile tip, immediately transfer 100 µL from the 10^{-3} microtube to the 10^{-4} microtube and mix as before.

f With a sterile tip, immediately transfer 100 µL from the 10^{-4} microtube to the 10^{-5} microtube and mix as before.

g With a sterile tip, immediately transfer 100 µL from the 10^{-5} microtube to the 10^{-6} microtube and mix as before.

3 At this point, each member of the student pair will work individually using the same diluted samples but different plates. You will each need 3 MRS plates.

a Label the 3 MRS plates with your name, then label them 10^{-5} through 10^{-7} and lay them out in sequence with the lids up.

b Be sure you know how to do a spread plate inoculation. If not, see Exercise 1-5 before continuing.

c Gently mix the contents of the 10^{-4} dilution tube and then transfer 100 µl to the 10^{-5} plate. Immediately proceed with the next step.

d Flame the alcohol off the glass rod (do not keep it in the flame and do not hold it over the alcohol beaker) and touch it to the agar surface (away from the inoculum) to cool it. Then spread the inoculum over the entire agar surface. When you are done, set the plate to the side; do not invert it yet.

e Using a fresh pipette tip for each plate, transfer 100 µL of the diluted samples to their appropriate plates as indicated below. Gently mix the contents of each tube before transferring. Also, spread the inoculum before transferring from the next tube.

100 µl of dilution tube 10^{-5} to the 10^{-6} plate

100 µl of dilution tube 10^{-6} to the 10^{-7} plate

f Allow the inocula to soak into the agar. Then, tape the lids on the plates and stack them.

g If you have enough anaerobic jars, one member of each group should incubate their plates anaerobically (see Exercise 2-8) while the partner incubates theirs aerobically. If anaerobic jars are limited, then student groups can combine plates in the jars. At the very least one set of plates should be incubated anaerobically. Incubate all plates at 35°C until next lab period.

Lab Two: Completion of Viable Count of Yogurt Culture and Beginning of Yogurt Isolate Identification

1 Working individually, examine the three plates.

a Find the plate that has between 30 and 300 colonies. Plates with more than 300 colonies are TMTC (too many to count). Record TMTC and then set the plate(s) aside. Those with less than 30 colonies are TFTC (too few to count) and may also be set aside after recording.

b Using the hand counter, count the number of colonies on the "countable" plate. Use a pen to mark colonies on the plate's bottom as you count each one.

c Calculate the density of bacteria in the yogurt sample using the equation below and record your answer in Table 1 on the data sheet, page 681. The units are CFU/g.

$$\text{Original Density} = \frac{\text{Number of Colonies}}{\text{Plate Dilution Factor}} =$$

d Collect data from all class dilution series and record these in Table 2 on the data sheet, page 682. Then, calculate the average CFU density from all samples. Calculate those incubated aerobically separately from those incubated anaerobically.

e Answer questions 1–6 related to CFU density on the data sheet, pages 683 and 684.

2 Still working individually, begin the identification process.

a Examine a plate with distinct colonies. It doesn't have to be a countable plate, nor does it have to be one the student inoculated. There should be as few as three and as many as five or six colony morphologies. Choose one of them (different than the other members at your table) and record its morphology in Table 3 on the data sheet, page 682.

b Transfer a portion of that colony to a lactobacilli MRS broth. Label it with your name and incubate it at 35°C. If it was taken from a plate incubated anaerobically, incubate it in an anaerobic jar. (Students with anaerobic isolates should put their MRS broth tubes in as few anaerobic jars as possible.) Otherwise, incubate it aerobically.

c Gram stain your colony. Use *E. coli* as a negative control. ***Note***: The organisms in yogurt are Gram-positive, thus the need for only a Gram-negative control. Many of them also decolorize easily, so you may see Gram-positives (especially rods) that are a mixture of pink and purple (Fig. 9.2). If they came from your plate and are not a contaminant, there is a very good chance they are Gram-positive. You are mostly looking for cell morphology and arrangement, anyway.

Lab Three: Continuation of Yogurt Isolate Identification

1 Continue working individually.

a Inoculate one of each fermentation tube (VP and all BCPs) listed in Table 9-2, page 677 from your pure culture. Be sure to label the tubes with the medium and your name.

b Incubate one MRS broth at 15°C and the other at 45°C. The remainder can be incubated at 35°C.

c Gram stain your pure culture using *E. coli* as your Gram-negative control. Does it look the same as your previous Gram stain?

Lab Four: Completion of Yogurt Isolate Identification

1 Read the test results.

a For the BCP tubes, yellow is positive (+) and purple is negative (−) for acid production. Be sure not to mix up the tubes! Acid from mannose is not the same as acid from mannitol.

b Read the MRS tube. Turbidity is positive, no turbidity is negative.

c Read the VP test (Exercise 5-4). Red is positive and no change is negative.

d When you have all the results, compare them to the results in Tables 9-1 and 9-2 to make a provisional identification of your isolate.

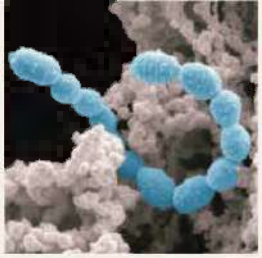
e Record your results in Table 4, page 683, and answer the question related to identification on the data sheet.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 9-3

Yogurt CFU Density of an Active and Live Culture and Identification of a Yogurt Unknown

OBSERVATIONS AND INTERPRETATIONS

1 Record the data from all three plates relevant to calculating yogurt CFU density. Then, use the equation to calculate it.

TABLE 1 **Lab Two Individual Results**

Plate Dilution Factor	Number of Colonies	Calculated CFU/g	Aerobic/Anaerobic

$$\text{Original Density} = \frac{\text{Number of Colonies}}{\text{Plate Dilution Factor}} = \_\_\_\_\_$$

2 Record the class results in the table below. Calculate and record the mean CFU density for aerobic and anaerobic plates.

TABLE 2 **Lab Two Class Results**

Student	Aerobic Countable Plate (CFU/g)	Student	Aerobic Countable Plate (CFU/g)
Mean Cell Density		Mean Cell Density	

3 Record your observations and test results for your yogurt isolate in the table below.

TABLE 3 **Lab Two Morphology Results**

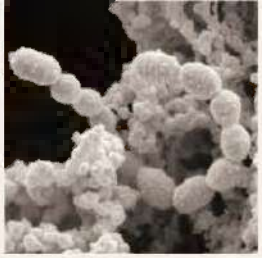
Cell Morphology and Arrangement (include a sketch)	Cell Dimensions	Colony Morphology

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 9-3

(continued)

4 Record your observations, test results, and the identification of your yogurt isolate in the table below.

TABLE 4 **Lab Four Yogurt Isolate Identification**

Growth at 15°C	Growth at 45°C	VP	Acid from Galactose	Acid from Mannitol	Acid from Mannose	Acid from Salicin	Acid from Sucrose

My yogurt isolate is: _____

QUESTIONS

1 *Does the use of CFU/g (as opposed to cells/g) underestimate or overestimate the number of cells in a sample?*

2 *Why doesn't the average number of cells in a CFU for a particular species affect the use of colonies as a source to start a pure culture?*

3 *Why were only three plate dilutions (10^{-5}, 10^{-6}, and 10^{-7}) used in this experiment?*

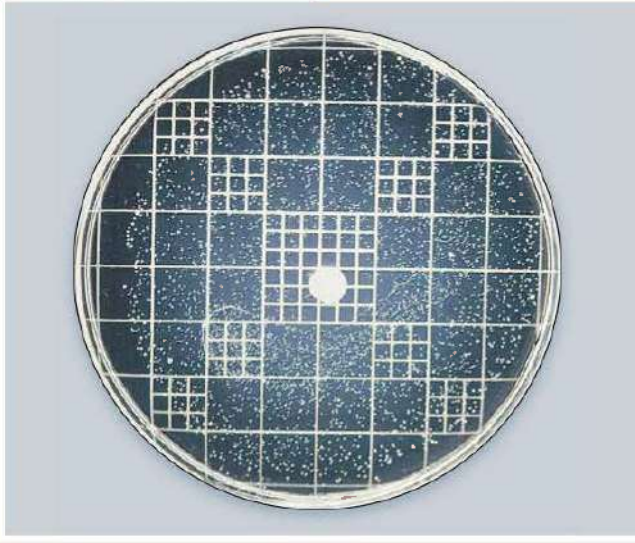
4 *What are sources of experimental error if your estimate of CFU/g for the yogurt sample was less than 10^8 CFU/g? Be very specific in describing them.*

5 *Suppose you repeat the viable count on several different batches of the same yogurt and you consistently get estimates that are close to 10^7 CFU/g. Would that manufacturer necessarily be mislabeling their product with the "Active & Live Cultures" seal? Explain.*

6 *Explain any difference in the mean CFU density for aerobic and anaerobic plates.*

SECTION 10

Microbial Genetics



In this unit you will be introduced to DNA experiments dealing with biotechnology (Exercises 10-1 through 10-4), mutation (Exercises 10-5 and 10-6), and molecular taxonomy (Exercise 10-7). First you will perform a simple extraction of *E. coli* DNA. The second exercise provides an example of how DNA can be digested into fragments and how those fragments can be put to use. As such, it is an important test in the fight against cancer. The third exercise incorporates into one exercise bacterial transformation, use of antibiotic selective media, genetic regulation, and genetic engineering. You will be transferring a specific gene into *E. coli* cells, selecting for only those cells that have been transformed successfully, then manipulating the environment so the organisms produce the gene product only when you want them to. The fourth exercise illustrates a sophisticated means of copying DNA *in vitro*.

In the next two exercises you will examine mutations—alterations of DNA. The first of these examines the effects of ultraviolet (UV) radiation on bacteria. It illustrates some characteristics of a particular *mutagen*—how it causes damage, factors affecting its impact on the cell, and how bacteria are able to repair that damage. The second mutation exercise—the Ames Test—illustrates a simple method of screening substances (commercial products) for mutagenicity, and in turn, carcinogenicity!

In the last exercise, you will see how molecular differences in *E. coli* strains, as detected by different susceptibility to bacteriophage infection, can be used as an aid to identification.

Extraction of DNA from Bacterial Cells[1]

EXERCISE 10-1

Theory

DNA extraction from cells is surprisingly easy and occurs in three basic stages.

1. A detergent (e.g., sodium dodecyl sulfate—SDS) is used to lyse cells and release cellular contents, including DNA.
2. This is followed by a heating step (at approximately 65°C–70°C) that denatures protein (including DNases that would destroy the extracted DNA) and other cell components. Temperatures higher than 80°C will denature DNA, and this is undesirable. A protease also may be added to remove proteins. (Other techniques for purification may be used, but these will not be included in this exercise.)
3. Finally, the water-soluble DNA is precipitated in cold alcohol as a whitish, mucoid mass (Fig. 10.1).

As an optional follow up to extraction, an ultraviolet spectrophotometer (Fig. 10.2) will be used to estimate DNA concentration in the sample by measuring absorbance at 260 nm, the optimum wavelength for absorption by DNA. An absorbance of A_{260nm} of 1 corresponds to 50 µg/mL of double-stranded DNA (dsDNA). To calculate the DNA concentration (**X**), use an equation of proportions:

$$\frac{\mathbf{X}\ \mu g/mL\ dsDNA}{Abs_{260}} = \frac{50\ \mu g/mL\ dsDNA}{Abs_{260}1.0}$$

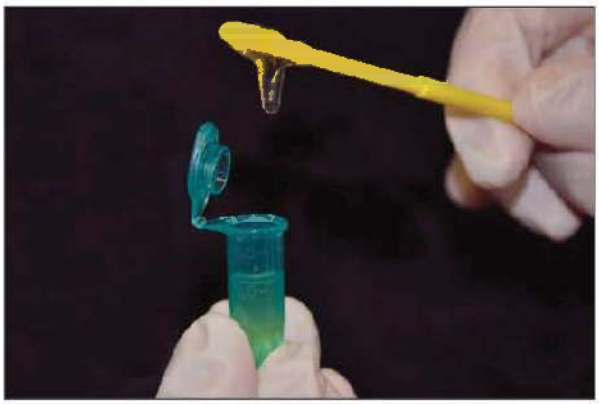
$$\mathbf{X}\ \mu g/mL\ dsDNA = 50\ \mu g/mL\ dsDNA \times Abs_{260}$$

Reading absorbance at 280 nm and calculating the following ratio can determine purity of the sample:

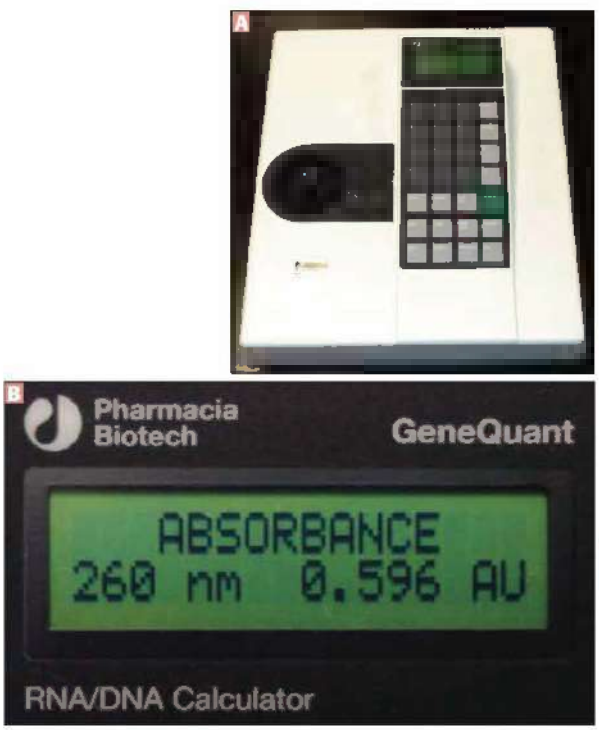
$$\text{Sample Purity} = \frac{Abs_{260\ nm}}{Abs_{280\ nm}}$$

If the sample is reasonably pure nucleic acid, the ratio will be about 1.8 (between 1.65 and 1.85). Because protein absorbs maximally at 280 nm, a ratio of less than 1.6 is likely because of protein contamination. If purity is crucial, the DNA extraction can be repeated. If the ratio is greater than 2.0, the sample is diluted and read again.

[1] Thanks to the following individuals who offered helpful suggestions for this protocol: Dr. Melissa Scott of San Diego City College; Allison Shearer of Grossmont College; Donna Mapston and Dr. Ellen Potter of Scripps Institute for Biological Studies; and Dr. Sandra Slivka of Miramar College.



10.1 Precipitated DNA ♦ This *E. coli* DNA has been spun onto the handle of a disposable inoculating loop.



10.2 Ultraviolet Spectrophotometer ♦ **(A)** A UV spectrophotometer can be used to determine DNA concentration. A quartz cuvette is shown in the sample port. **(B)** This specimen has an A_{260nm} of 0.596. Because an A_{260nm} of 1.0 is equal to 50 µg/mL of dsDNA, this specimen has a concentration of 29.8 µg dsDNA/mL. Absorbance also can be used to determine purity of the sample. A relatively pure DNA sample will have an A_{260nm}/A_{280nm} value of approximately 1.8.

Application

Extraction of DNA is a starting point for many lab procedures, including DNA sequencing and cloning.

In This Exercise

You will extract DNA from *E. coli*. To improve yield, you first will concentrate an *E. coli* broth culture. The actual extraction involves cell lysis, denaturation of protein, and precipitation of the DNA in alcohol. Following extraction, an optional procedure may be used to determine DNA yield and the purity of your extract.

✓ Materials

Per Pair of Students

- ☐ Overnight culture of *Escherichia coli* in Luria-Bertani broth (young cultures work best)
- ☐ Water bath set to 65°C
- ☐ Microtube floats
- ☐ Ice bath (small cups with crushed ice work well)
- ☐ 300 µL 10% sodium dodecyl sulfate (SDS)
- ☐ 50 µL 20 mg/mL Proteinase K solution (stored in freezer between uses) (*Note:* Meat tenderizer is an inexpensive substitute, though it may result in DNA hydrolysis if too much is used.)
- ☐ 300 µL 1.0 M sodium acetate solution (pH = 5.2)
- ☐ 3 mL 1× tris-acetate-EDTA (TAE) buffer (dilute 10× TAE 9 + 1)
- ☐ 2 mL 90% isopropanol (stored in a freezer or an ice bath)
- ☐ Two calibrated disposable transfer pipettes
- ☐ 100–1,000 µL digital pipettor and tips
- ☐ Container for tip disposal
- ☐ 25 mL centrifuge tube
- ☐ Tabletop centrifuge
- ☐ Minicentrifuge
- ☐ Disposable inoculating loop
- ☐ Two microtubes (at least 1.5 mL in volume)
- ☐ (Optional) vortex mixer
- ☐ (Optional) ultraviolet spectrophotometer
- ☐ (Optional) two quartz cuvettes

Medium Recipe

Luria-Bertani Broth

• Tryptone	10.0 g
• Yeast extract	5.0 g
• NaCl	10.0 g
• Distilled or deionized water	1.0 L

pH 7.4 at 25°C

Procedure

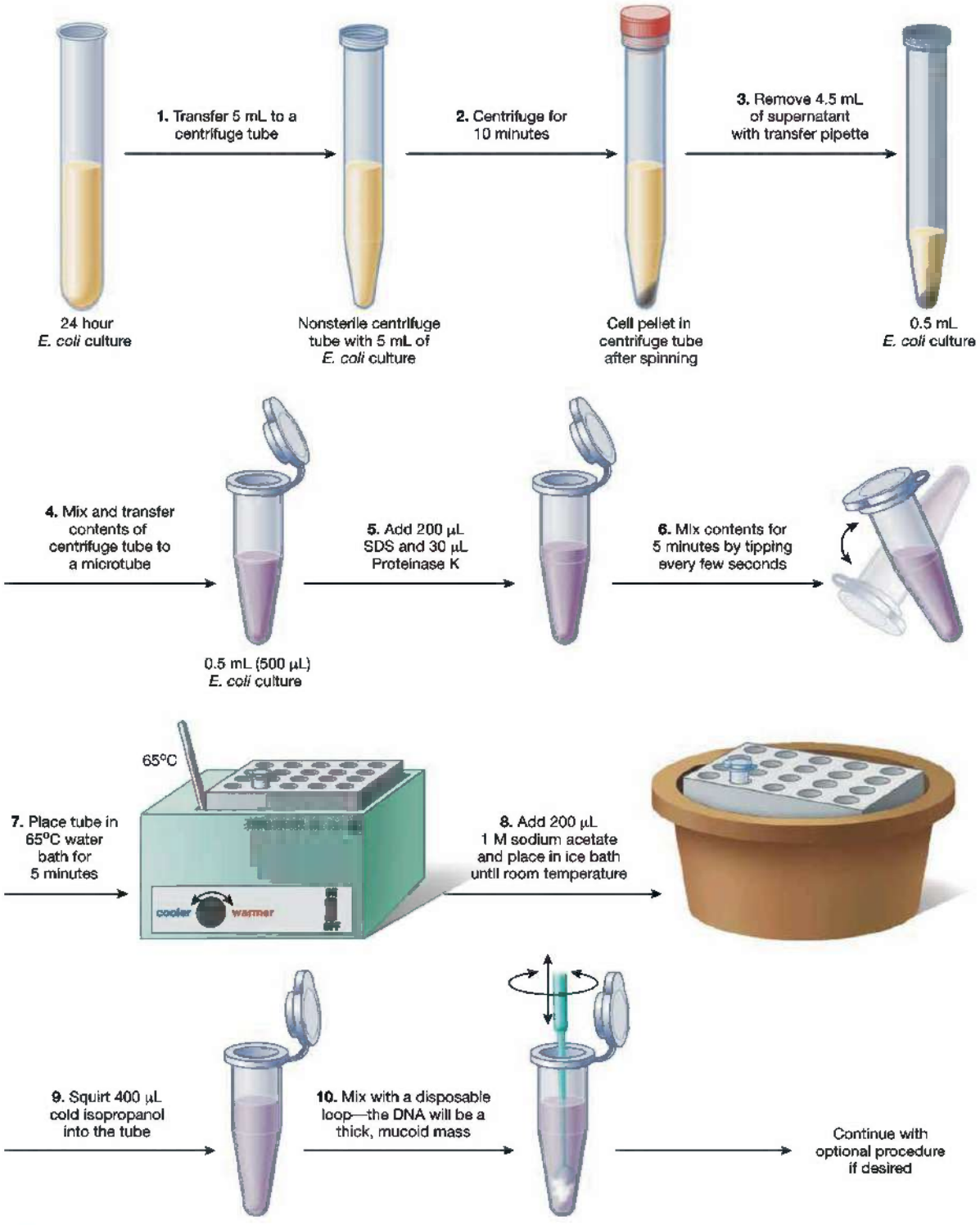
Refer to the procedural diagram in Figure 10.3 as you read and follow this protocol.

1. Obtain the *E. coli* culture. Mix the suspension until uniform turbidity is seen, then transfer 5 mL to a clean, nonsterile centrifuge tube.
2. Spin the 5 mL sample in the tabletop centrifuge slowly for 10 minutes to produce a cell pellet.
3. After spinning and without disturbing the pellet, remove 4.5 mL of the supernatant (the liquid above the pellet) with a transfer pipette. Dispose of the supernatant in the original culture tube.
4. Transfer the remaining 0.5 mL to a nonsterile microtube.
5. Add 200 µL of 10% SDS to the *E. coli*.
6. Add 30 µL of 20 mg/mL Proteinase K solution, or a "pinch" of meat tenderizer.
7. Close the cap and gently mix the tube for 5 minutes by tipping it upside down every few seconds.
8. Place the tube in a float and incubate in a 65°C water bath for 5 minutes.
9. Add 200 µL 1 M sodium acetate solution and mix gently.
10. Place the tube in the ice bath until it is at or below room temperature.
11. When cooled, squirt 400 µL of cold 95% isopropanol into the preparation. (If there are bubbles on the surface, remove them with a nonsterile transfer pipette prior to adding the isopropanol.)
12. Use either end of a disposable loop to mix the preparation, moving in and out and turning occasionally. A glob of mucoid DNA will begin to appear as you mix and will adhere to the loop.

Optional Procedure
(if your lab has an ultraviolet spectrophotometer)

Refer to the procedural diagram in Figure 10.4 as you read and complete the following protocol.

1. Remove the DNA from the original microtube and transfer it to a second microtube, using the loop.
2. Resuspend the DNA in 1,000 µL isopropanol using a nonsterile transfer pipette, and then spin it in a minicentrifuge for a few seconds. The DNA should be at the bottom of the tube.
3. With a nonsterile transfer pipette, remove the supernatant and allow the DNA pellet to air-dry.
4. Resuspend the dried DNA in 1,000 µL of TAE buffer. Mix vigorously to dissolve the DNA in the TAE. This mixing may be done by hand, or a vortex mixer may be used.


1. Transfer 5 mL to a centrifuge tube
2. Centrifuge for 10 minutes
3. Remove 4.5 mL of supernatant with transfer pipette
24 hour E. coli culture
Nonsterile centrifuge tube with 5 mL of E. coli culture
Cell pellet in centrifuge tube after spinning
0.5 mL E. coli culture
4. Mix and transfer contents of centrifuge tube to a microtube
5. Add 200 µL SDS and 30 µL Proteinase K
6. Mix contents for 5 minutes by tipping every few seconds
0.5 mL (500 µL) E. coli culture
65°C
7. Place tube in 65°C water bath for 5 minutes
cooler
warmer
8. Add 200 µL 1 M sodium acetate and place in ice bath until room temperature
9. Squirt 400 µL cold isopropanol into the tube
10. Mix with a disposable loop—the DNA will be a thick, mucoid mass
Continue with optional procedure if desired

10.3 Procedural Diagram for Bacterial DNA Extraction

5 Transfer the suspended DNA solution into a quartz cuvette.

6 Prepare a second cuvette containing 1,000 µL of TAE as a blank.

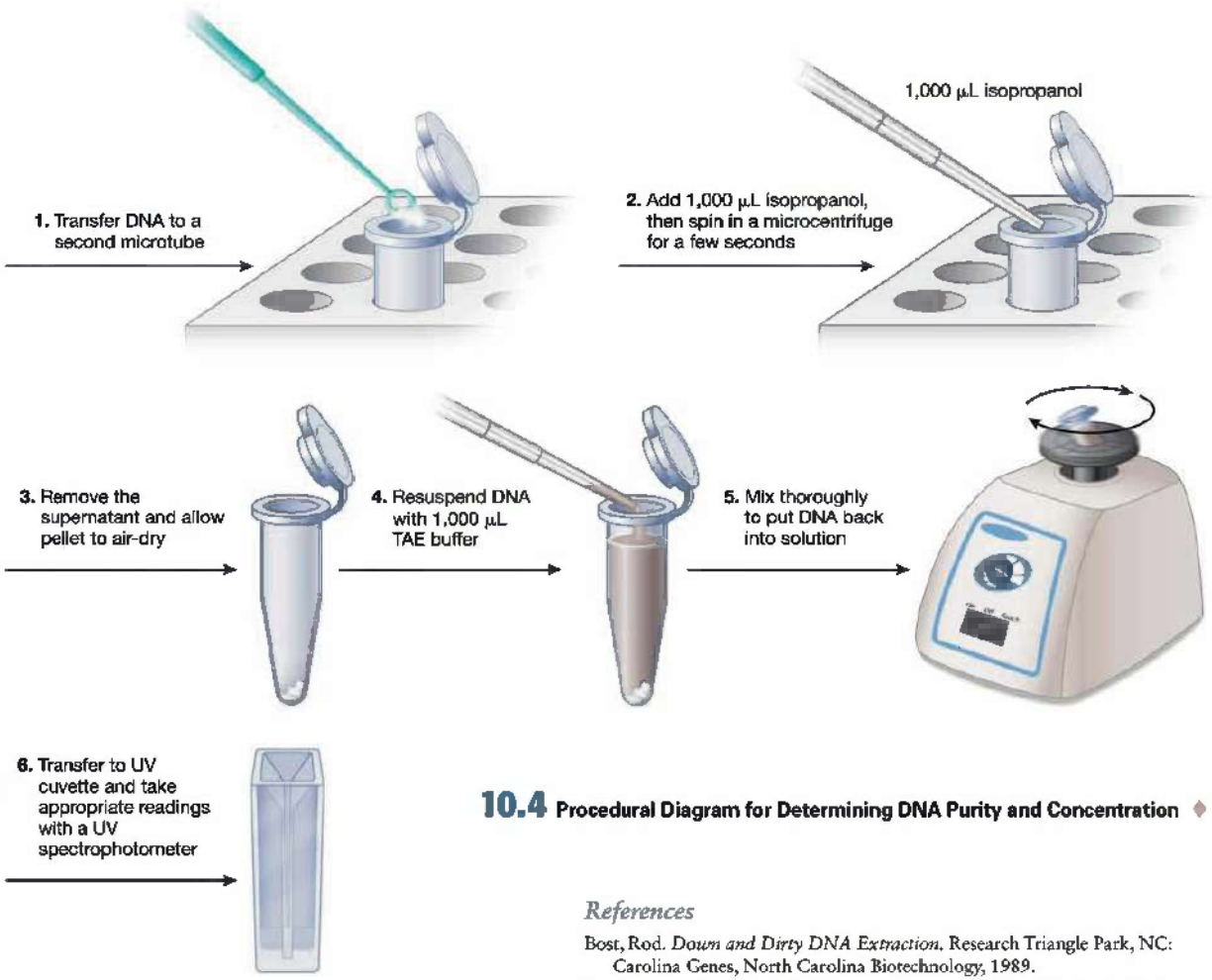
7 Set the spectrophotometer to 260 nm wavelength. Follow the instructions for your UV spectrophotometer to check the absorbance of the extracted DNA, and record on the data sheet, page 691.

8 Set the spectrophotometer to 280 nm wavelength. Follow the instructions for your UV spectrophotometer to check the absorbance of the extracted DNA, and record on the data sheet.

9 Calculate the probable purity of your extracted DNA sample using the formula provided, and record on the data sheet.

10 If desired, absorbencies at other wavelengths may be taken to produce an absorption spectrum for DNA. Suggested wavelengths are: 220 nm, 240 nm, 300 nm, and 320 nm, in addition to the measurements for 260 and 280 nm taken above. Record these on the data sheet.

11 If step 10 was done, plot the absorption spectrum (Absorption versus Wavelength) of the DNA sample on the data sheet.


1. Transfer DNA to a second microtube
1,000 µL isopropanol
2. Add 1,000 µL isopropanol, then spin in a microcentrifuge for a few seconds
3. Remove the supernatant and allow pellet to air-dry
4. Resuspend DNA with 1,000 µL TAE buffer
5. Mix thoroughly to put DNA back into solution
6. Transfer to UV cuvette and take appropriate readings with a UV spectrophotometer

10.4 Procedural Diagram for Determining DNA Purity and Concentration

10

References

Bost, Rod. *Down and Dirty DNA Extraction.* Research Triangle Park, NC: Carolina Genes, North Carolina Biotechnology, 1989.

Davis, Leonard G., Mark D. Dibner, and James F. Battey. *Basic Methods in Molecular Biology.* New York: Elsevier Science Publishing, 1986.

Freifelder, David. Pages 504–505 in *Physical Biochemistry,* 2nd ed. New York: W. H. Freeman and Co, 1982.

Kreuzer, Helen and Adrianne Massey. *Recombinant DNA and Biotechnology—A Guide For Teachers,* 2nd ed. Washington, DC: ASM Press, 2001.

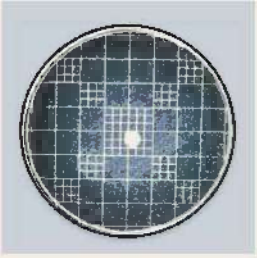
Zyskind, Judith W. and Sanford I. Bernstein. *Recombinant DNA Laboratory Manual.* San Diego, CA: Academic Press, 1992.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 10-1

Extraction of DNA from Bacterial Cells

OBSERVATIONS AND INTERPRETATIONS

1 In the following table, record the absorbance values for the wavelengths used.

Wavelength (nm)	Absorbance
220 (optional)	
240 (optional)	
260	
280	
300 (optional)	
320 (optional)	

2 Calculate the DNA concentration in your sample. Be sure to take any dilutions into account.

3 Determine the probable purity of your sample using the equation:

$$\frac{\text{Absorbance}_{260nm}}{\text{Absorbance}_{280nm}}$$

4 Plot the absorption spectrum (Absorption versus Wavelength) of the DNA sample if absorbance at additional wavelengths was determined.

QUESTIONS

1 *What is the importance of heating the cell lysate?*

2 *Why does the extraction work better with cold 95% isopropanol that with room-temperature 95% isopropanol?*

3 *Based on your results, what wavelength gives maximum absorption of DNA? Does this match the accepted wavelength for maximum absorption? If not, suggest reasons for the discrepancy.*

Restriction Digest

EXERCISE 10-2

Theory

Restriction enzymes are **endonucleases** produced by many *Bacteria* and *Archaea* whose functions are to repair the cell's own DNA and protect it against foreign DNA. Each restriction enzyme has a different **restriction (recognition) site**, a short sequence of 4 to 8 nucleotides with a unique pattern to which it attaches. Once bound to the restriction site, the enzyme catalyzes hydrolysis of covalent bonds in both sugar-phosphate backbones, allowing removal of damaged segments or, in the case of invading viral DNA, disabling it before it can damage the cell.

The name of a restriction enzyme comes from a combination of the genus, specific epithet, and strain (when applicable) of the organism that produces it, and a Roman numeral to indicate its order of identification, because a single organism often synthesizes many different ones. Below is the recognition sequence of *Eco*RI, the first (I) restriction enzyme isolated from the RY13 strain of *Escherichia coli*.

5'–GAATTC–3'
3'–CTTAAG–5'

Methylation is a process that protects a cell's DNA from damage caused by its own endonucleases. **Methylase enzymes** transfer methyl groups ($-CH_3$) to restriction sites and render them unfit substrates for the cell's arsenal of restriction enzymes. The following diagram illustrates how the enzyme *Eco*RI methylase modifies the restriction site for the above-mentioned restriction enzyme *Eco*RI. Together these enzymes comprise the *Eco*RI restriction-modification system.

CH_3
|
5'–GAATTC–3'
3'–CTTAAG–5'
|
CH_3

In the case of the *Eco*RI restriction-modification system, the restriction enzyme and the methylase are separate enzymes, but in other cases a multi-subunit enzyme performs both restriction digestion and methylation.

Most recognition sites for endonucleases are **palindromes**. That is, both strands of DNA have the same nucleotide sequence when read from the 5' (or 3') end. Each enzyme cuts the DNA backbone at a specific location in the sequence. For example, *Eco*RI cuts (cleaves) its six-base recognition sequence between the guanine and adenine in each strand to produce the following pattern:

5'–G AATTC–3'
3'–CTTAA G–5'

Notice that the ends of the resulting cut DNA contain single-stranded overhangs consisting of four bases. Molecular biologists often refer to these overhangs as "sticky ends" because fragments with complementary overhangs will easily **ligate** (stick by base pairing) together. This characteristic is particularly useful for cloning genes or assembling DNA fragments.

Biotechnology and genetic engineering often involve the use of restriction enzymes to produce pieces of DNA with complementary ends that allow the researcher to assemble new configurations of DNA for a variety of experimental and therapeutic purposes. A four-base overhang, however, is only one possibility. Many cleavage patterns are possible with restriction enzymes. *Sma*I, the first restriction enzyme isolated from *Serratia marcescens*, produces blunt ends by cutting through both strands of the DNA backbone in the middle of the recognition sequence as shown in the following pattern.

5'–CCC GGG–3'
3'–GGG CCC–5'

Additionally, some enzymes bind to and cleave multiple variations of a sequence. For example, *Bgl*I, from the bacterium *Bacillus globigii*, recognizes 625 variations of the following sequence:

5'–GCCNNNNNGGC-3'
3'–CGGNNNNNCCG-5'

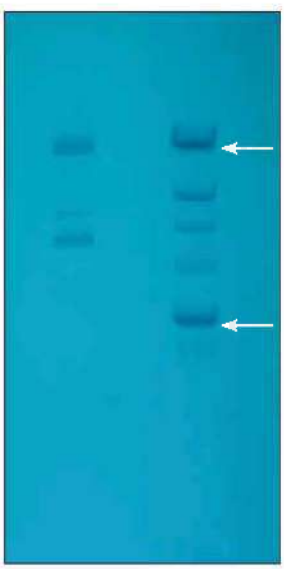
where N can be A,T, G, or C. The enzyme recognizes the sequences flanking the variable segment, attaches to the DNA, and facilitates the reaction only as long as the nucleotides are spaced as shown. Table 10-1 lists a few examples of restriction enzymes and cleavage patterns.

TABLE **10-1** **Select Restriction Endonucleases and Their Recognition Sequences**

Enzyme	Source Organism	Recognition Sequence	Digest Pattern
*Apa*LI	*Acetobacter pasteurianus*	5'–GTGCAC–3' 3'–CACGTG–5'	5'–G TGCAC–3' 3'–CACGT G–5'
*Bam*HI	*Bacillus amyloliquefaciens*	5'–GGATCC–3' 3'–CCTAGG–5'	5'–G GATCC–3' 3'–CCTAG G–5'
*Eco*RI	*Escherichia coli*	5'–GAATTC–3' 3'–CTTAAG–5'	5'–G AATTC–3' 3'–CTTAA G–5'
*Kpn*I	*Klebsiella pneumoniae*	5'–GGTACC–3' 3'–CCATGG–5'	5'–GGTAC C–3' 3'–C CATGG–5'
*Pvu*I	*Proteus vulgaris*	5'–CGATCG–3' 3'–GCTAGC–5'	5'–CGAT CG–3' 3'–GC TAGC–5'
*Sau*3A	*Staphylococcus aureus*	5'–GATC–3' 3'–CTAG–5'	5'– GATC–3' 3'–CTAG –5'
*Sma*I	*Serratia marcescens*	5'–CCCGGG–3' 3'–GGGCCC–5'	5'–CCC GGG–3' 3'–GGG CCC–5'

The activity of restriction enzymes is measured in *units* (u), where one unit of a restriction endonuclease is defined as the amount of enzyme required to digest 1 µL of a DNA test sample in 1 hour at 37°C in a total reaction volume of 50 µL.

Once the DNA has been cut by the enzyme, the resulting fragments must be separated and stained in order to be observed. To separate DNA fragments of different sizes and allow visualization and subsequent recovery, agarose gel electrophoresis is frequently used in conjunction with restriction enzymes. Many variables can affect migration patterns of DNA fragments, including shape, size, charge, and a tendency of some fragments to form attachments to other molecules. Generally speaking, however, small DNA fragments (that is, fewer base pairs, or bp) will migrate faster and farther than large fragments (Fig. 10.5 and the standard curve shown on the data sheet, page 701). Simply looking at the bands on the gel will not tell you the size of the fragments, but running a control sample of DNA fragments of known sizes in the gel along with your samples allows construction of a standard curve that can be used to determine unknown fragment sizes. Agarose gel electrophoresis is explained more fully in Appendix G.



10.5 Restriction Digest Gel ◆ This gel contains two lanes from a restriction digest. The bands on the right are from the DNA standard fragments (MW markers). The bands on the left are from a DNA sample cleaved by one enzyme. In this photo the wells are at the top and migration was downward. The arrows indicate the "bottom" of a band and is the point to which you measure in determining migration distance.

Application

Restriction enzymes are used extensively in genetic engineering, most frequently to produce smaller DNA polynucleotides with known "sticky ends." Subsequently, a fragment containing a desired gene can be inserted into other DNA that is cut to produce the same sticky ends, because they will naturally and complementarily base pair and ligate easily. The resulting recombinant DNA molecule can then be introduced into a cell that produces the desirable gene product.

In This Exercise

You will be given one type of DNA (the pUC19 cloning vector plasmid) and up to three restriction enzymes (*Apa*LI, *Bam*HI, and *Pvu*I) with which to run a restriction endonuclease enzyme digestion. When your digestion is completed, you will electrophorese your samples along with DNA standards (molecular weight marker controls) in an agarose gel and use the band migration distances to determine the resulting DNA fragment sizes. You then will compare your experimentally determined fragment sizes to your predictions from the pUC19 cloning vector plasmid map.

✓ Materials

For Digestion Reactions:

Per Class

- □ 37°C water bath

Per Student Group

- □ Ice bath
- □ Microcentrifuge
- □ (Optional) vortex mixer
- □ Floating tube rack
- □ Micropipettors and sterile tips to handle volumes between 1 µL and 50 µL
- □ Tip disposal container
- □ Three 1.5 mL reaction tubes each containing 5 µg of a 1 µg/µL solution of pUC19
- □ *Apa*LI restriction enzyme 10–20 units/µL (on ice)
- □ *Bam*HI restriction enzyme 10–20 units/µL (on ice)
- □ *Pvu*I restriction enzyme 10–20 units/µL (on ice)
- □ 10× reaction buffer for *Apa*LI
- □ 10× reaction buffer for *Bam*HI
- □ 10× reaction buffer for *Pvu*I
- □ Enzyme grade ultrapure water

For Agarose Gel Electrophoresis (Appendix G):

Per Class

- □ 50°C large water bath

Per Student Group

- □ Gel casting tray, two rubber dams or tape, and with an eight-well comb (wells should accommodate 60 µL each)
- □ Electrophoresis running chamber
- □ 1× TAE (Appendix H)
- □ Powdered agarose
- □ Balance and weigh paper
- □ 250 mL flask with foil cover
- □ Magnetic stir bar
- □ Magnetic hot plate
- □ 6× DNA loading dye (Appendix H)
- □ DNA standards (a molecular weight marker that can identify bands between 0.4 kb and 3 kb; 2-log or 1 kb DNA Ladder is suggested)
- □ Micropipettors and sterile tips to handle volumes between 10 µL and 60 µL
- □ Electrophoresis power supply
- □ 0.1× TAE (Appendix H)
- □ DNA staining solution (Appendix H)

Procedure

(Overall time ~2 to 16 hours)

Preparation and Digestion Reaction (~1 hour)

Follow the procedural diagram in Figure 10.6.

1 Using the table on the data sheet, page 699, plan your digestion reactions. (*Note*: Your instructor may assign you only one enzyme, or all three, depending on resources available. Even if you are doing only one reaction, you should work out the reaction recipes and predicted results for all three.) Calculate the amount of each reagent needed for each 50 µL digestion reaction. In each case, the amount of water is calculated by subtracting the sum of all the other reagents from the total 50 µL reaction volume.

a The enzyme buffer is supplied at a 10× concentration and must be at a final concentration of 1×.

$$C_1V_1 = C_2V_2$$

C_1 is the stock concentration of the reagent in molar, percent, or X stock

C_2 is the final concentration of the reagent in the reaction in the same units as C_1

V_1 is the amount of the stock you need (this is what you are solving for)

V_2 is the final volume of the reaction, in this case 50 µL

Note: This is the same equation as $V_1D_1 = V_2D_2$ used in Exercise 6-1 except that in this case you are solving for *concentration* instead of *dilution factor*. Proportions are proportions!

b The enzymes are supplied at 10 or 20 units/µL but regardless of concentration you will be using 1 µL per reaction.

c The DNA is supplied at 1 µg/µL and you will be digesting 5 µg per reaction (5 µL of DNA is already present in each reaction tube).

2 Obtain your reagents and set up the reactions according to your plan. Bear in mind that the volumes being used are very small and care must be taken to pipette accurately (Appendix D). Use a clean pipette tip between each transfer. Keep the enzymes on ice at all times and add the enzymes to your reactions last, that is, after the water and buffer have already been added to the DNA in the tube. When adding the enzyme, pipette up and down gently a few times to ensure delivery because the enzyme solution is thick and tends to stick to the pipette tip.

3 Cap the reaction tubes tightly and tap or vortex them GENTLY to mix the ingredients.

1. Place all tubes except ApaLI, PvuI, and BamHI in rack and label properly. Keep the enzymes on ice.
Water
ApaLI Buffer
BamHI Buffer
PvuI Buffer
Rxn 1 DNA
Rxn 2 DNA
Rxn 3 DNA
ApaLI
BamHI
PvuI
2. Add ingredients (from Table 1 on the data sheet, page 699) to reaction tubes. Add enzymes last.
Rx 1
Rx 2
Rx 3
3. Mix all tubes gently.
4. Force all ingredients to the bottom of the tube by spinning 20 seconds.
37°C
BEP-MJPLab. Inc.
cooler
warmer
5. Incubate all tubes at 37°C for 30 minutes.
6. Add 10 µL 6x gel loading dye to each tube. Mix gently. Follow the instructions for electrophoresis (Appendix G).
Rx

10.6 Procedural Diagram for Restriction Digest ♦

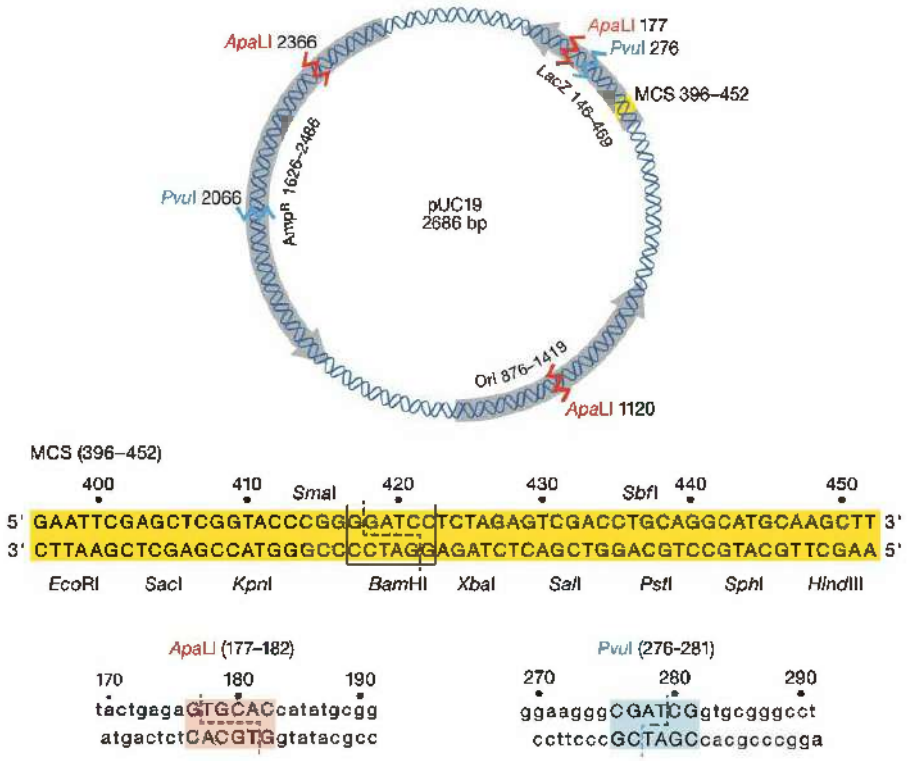
4 Spin the tubes in a microcentrifuge for 20 seconds to force the mixture to the bottom of the tube. Be sure to balance the centrifuge before starting the spin cycle.

5 Place the tubes in a floating tube rack and incubate them in the 37°C water bath for 30 minutes.

6 During incubation, prepare your agarose gel according to the instructions in Appendix G, Module A.

7 Once the gel has been poured and while you wait for it to solidify, calculate the sizes of DNA fragments that will be generated with each enzyme using the information provided in the pUC19 map in Figure 10.7. Record your predictions on the data sheet.

8 When the digestion is complete, proceed to the next step, or freeze the reactions for storage until the next lab period.



10.7 pUC19 Plasmid DNA Cloning Vector ◆ This diagram depicts a map of the 2686 base pair DNA plasmid called pUC19. Some of the available restriction enzyme recognition sequence positions are labeled. Numbers indicate the nucleotide base position on the plasmid.

Agarose Gel Electrophoresis

(20 minutes to 2 hours, depending on gel size and voltage used)

1. Using a different pipette tip for each transfer, add 10 µL of 6× DNA loading dye to each digestion reaction tube.
2. Cap the reaction tubes tightly and tap or vortex them GENTLY to mix the ingredients.
3. Spin the tubes in a microcentrifuge for 20 seconds to force the mixture to the bottom of the tube. Be sure to balance the centrifuge before starting the spin cycle.
4. Record the well position in the gel of each sample and load all 60 µL of each reaction mixture into its assigned well. Use a different pipette tip for each.
5. Load 5 µg of a DNA ladder molecular weight marker in loading dye in the well adjacent to the last sample. This sample will serve as a control for the experiment.
6. Run your gel according to the instructions in Appendix G, Module B.

Gel Staining and Destaining

(4 hours to overnight)

1. Stain and destain the agarose gel according to the instructions in Appendix G, Module C.
2. Measure the migration distance of each band and complete the table and graph on the data sheet. Compare your results to the fragment sizes that you predicted from the pUC19 map.

10

References

Krebs, Jocelyn E., Elliott S. Goldstein, and Stephen T. Kilpatrick. Section 3.2 in *Lewin's GENES XI*, 11th ed. Burlington, MA: Jones and Bartlett Learning, 2012.

Madigan, Michael T., John M. Martinko, David Stahl. Chap. 11 in *Brock Biology of Microorganisms*, 13th ed. Boston: Benjamin Cummings, 2010.

National Center for Biotechnology Information. "Cloning vector pUC19c, complete sequence." Available online. URL: http://www.ncbi.nlm.nih.gov/nuccore/L09137.

Nelson, David L. and Michael M. Cox, Chaps. 8 and 9 in *Lehninger Principles of Biochemistry*, 6th ed. New York: W.H. Freeman, 2012.

New England BioLabs. "Optimizing Restriction Endonuclease Reactions." Available online. URL: https://www.neb.com/protocols/2012/12/07/optimizing-restriction-endonuclease-reactions.

New England BioLabs. "pUC19 DNA: Location Sites." Available online. URL: https://www.neb.com/~/media/NebUs/Page%20Images/Tools%20and%20Resources/Interactive%20Tools/DNA%20Sequences%20and%20Maps/pUC19_sites.pdf.

New England BioLabs. "pUC19 GenBank Accession #: L09137." Available online. URL: https://www.neb.com/~/media/NebUs/Page%20Images/Tools%20and%20Resources/Interactive%20Tools/DNA%20Sequences%20and%20Maps/pUC19_map.pdf

Roberts, Richard J. "Restriction and modification enzymes and their recognition sequences." *Nucleic Acids Research* 8, no. 1 (January 1980): 197.

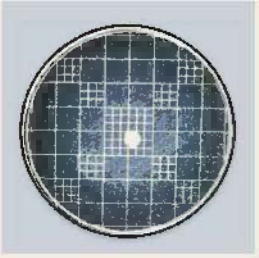
Watson, James D., Richard M. Meyers, and Amy A. Caudy. Chap. 5 in *Recombinant DNA: Genes and Genomes–A Short Course*, 3rd ed. Long Island, NY: Cold Spring Harbor Laboratory Press, 2007.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 10-2

Restriction Digest

OBSERVATIONS AND INTERPRETATIONS

1 Plan your digestion reaction mixtures. Reaction 1 has been calculated for you as a guide. First, calculate the buffer and enzyme volumes. Then, subtract their combined volumes from the desired final 50 µL total volume to determine the volume of water to be used. Record your answers in the following table.

Table 1 **Digestion Reaction Planner**

Reagent	Reaction 1	Reaction 2	Reaction 3
pUC19 DNA (1 µg/µL)	5 µL	5 µL	5 µL
Water	39 µL		
10× Buffer for *Apa*LI	5 µL	–	–
10× Buffer for *Bam*HI	–		–
10× Buffer for *Pvu*I	–	–	
*Apa*LI (10 U/µL)	1 µL	–	–
*Bam*HI (20 U/µL)	–		–
*Pvu*I (10 U/µL)	–	–	
Total Volume	**50 µL**	**50 µL**	**50 µL**

Always add the enzymes last after all the other reagents have been added.

2 Predict your results. Use the map of the pUC19 cloning vector plasmid in Figure 10.7 to calculate the expected fragment sizes that will be generated when the DNA is cut by the specified enzyme. Record your answers in kilobase pairs (kb) of DNA. For example, a fragment that is calculated to be 890 base pairs long would be indicated as 0.89 kb.

*Apa*LI fragment lengths:

*Bam*HI fragment lengths:

*Pvu*I fragment lengths:

3 Following gel electrophoresis, measure the migration distances for some of the DNA standard fragments and enter your data in the right-hand column in the following table. For consistency, measure from the bottom[1] of the well to the bottom of each band (Fig. 10.5). It is best not to include the largest or smallest fragments but do include at least five, and up to 10, fragments that span a range of sizes. For example, if you are using the 2-log DNA Ladder shown in the illustration below, the 0.5 kb, 0.8 kb, 1.0 kb, 1.5 kb, 2.0 kb, 3.0 kb, 4.0 kb and 6.0 kb fragments are likely easily visible and should produce good data for generating your standard curve.

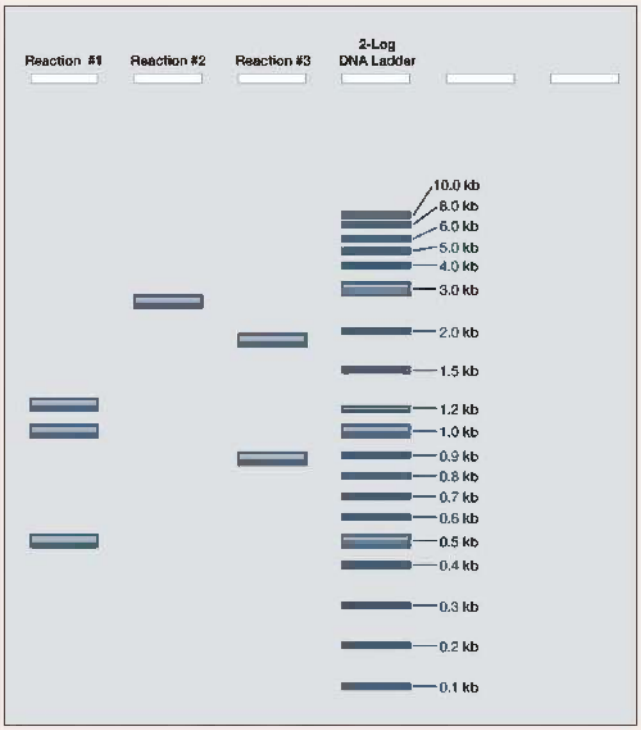
Table 2 **Fragment Migration Distances and Sizes**

Reaction #1		Reaction #2		Reaction #3		DNA Standards	
Distance (cm)	Size (kb)	Distance (cm)	Size (kb)	Distance (cm)	Size (kb)	Distance (cm)	Size (kb)

4 Measure the migration distance of each band in each lane from your digestion reactions in the same manner as described in step 3. Enter the data in the left-hand side of each column in Table 2.

5 Using the migration distance data in Table 2 and the base-pair length data of the DNA standards, construct a standard curve on the semilogarithmic graph paper provided on page 704. Refer to the example of a standard curve on page 701 as needed. Do not connect the data points; draw a straight trend line through them as centrally located as possible.

6 Use the standard curve and the data from the table in step 3 to determine the sizes of the digestion reaction fragments. To do this, locate the migration distance on the x-axis and draw a straight vertical line from that point up to the standard curve. Then draw a horizontal line from that point of intersection with the standard curve to the y-axis. The point where the horizontal line intersects the y-axis is the number of base pairs in the DNA fragment. Enter this data in the right-hand side of each column in Table 2 and compare them with your predicted sizes from step 2.



Sample Restriction Digestion Gel ♦ This drawing illustrates base pair sizes of the 2-Log DNA Ladder standard and three hypothetical restriction digests. This ladder is one suggested for use in this lab. Consult with your instructor for DNA standards sizes if you used a different ladder.

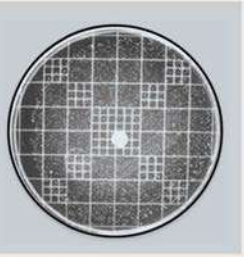
[1] The bottom of a well or band assumes the gel is being held vertically, so it represents the entry point into the gel out of the well and the leading edge of the bands.

Name _____

Date _____

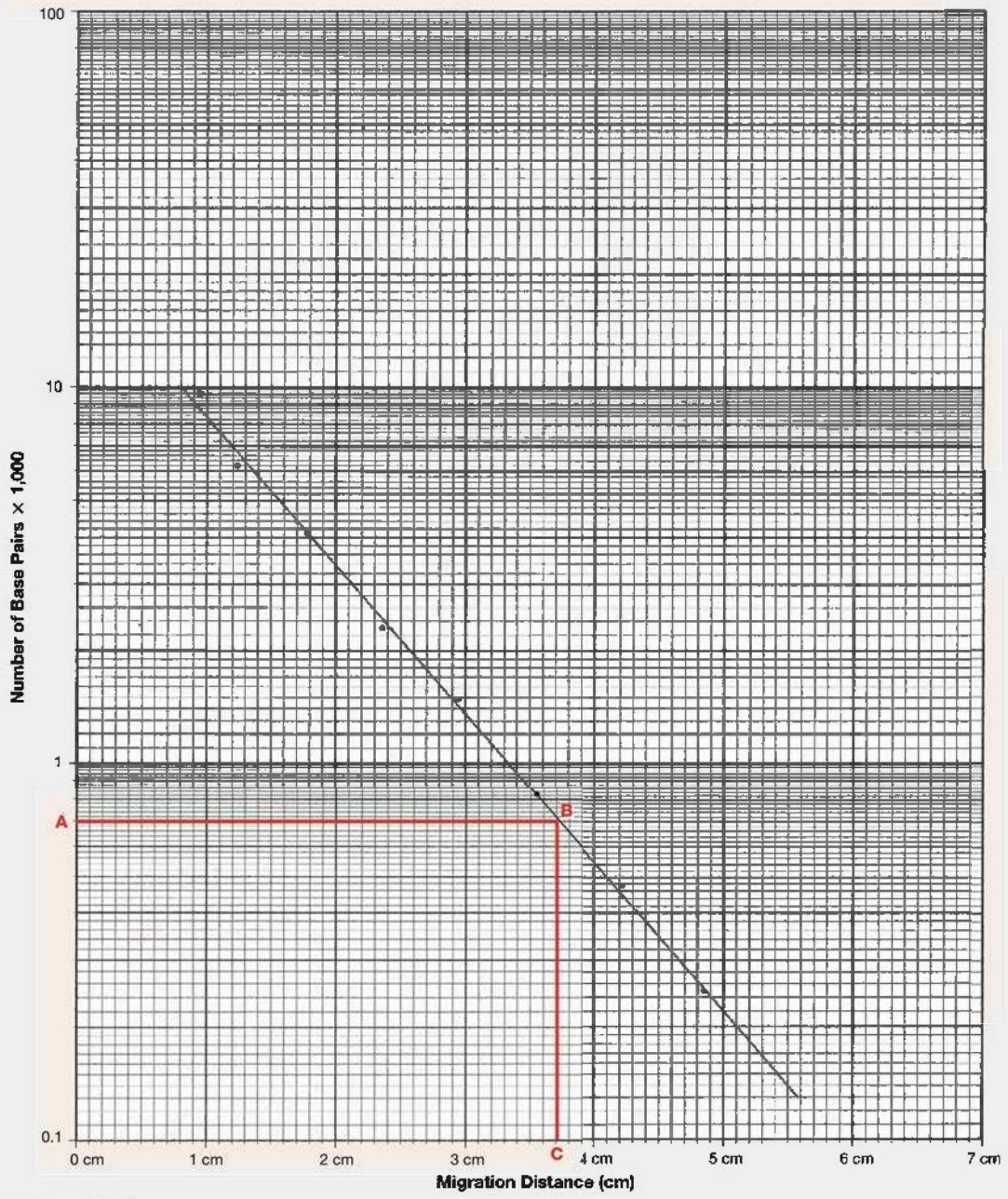
Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 10-2

(continued)



Standard Curve ◆ This example of a standard curve demonstrates how to determine base-pair sizes represented by the bands in your gel. Assume that a band, measured from the bottom of the well to the bottom of the band, had migrated 3.7 cm. A vertical line is drawn from the 3.7 cm point on the x-axis (A) to the diagonal line (standard curve) and then from that intersection (B), a horizontal line is drawn to the y-axis. The point where the horizontal line intersects the y-axis (C) reveals the fragment size in number of base pairs. The DNA fragments in this example contained 700 base pairs, or 0.7 kb.

QUESTIONS

1 *Did your observed fragment sizes from the restriction digests of pUC19 DNA match your predicted fragment sizes calculated from the plasmid map? If not, what factors do you think might have contributed to any discrepancies?*

2 *It was suggested that the largest and smallest fragments from your DNA standards be excluded from the data used to generate your standard curve in order to produce a more accurate curve. Why might this be so?*

3 *Which restriction enzyme is likely to produce more fragments with any given piece of DNA, one with a 4 base-pair recognition site or one with an 8 base-pair site? Explain your answer.*

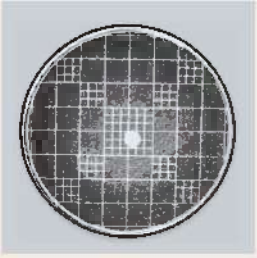
4 *Suppose you mistakenly made your agarose gel with tenfold more agarose than your recipe called for. How do you think this would affect the migration of the DNA?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



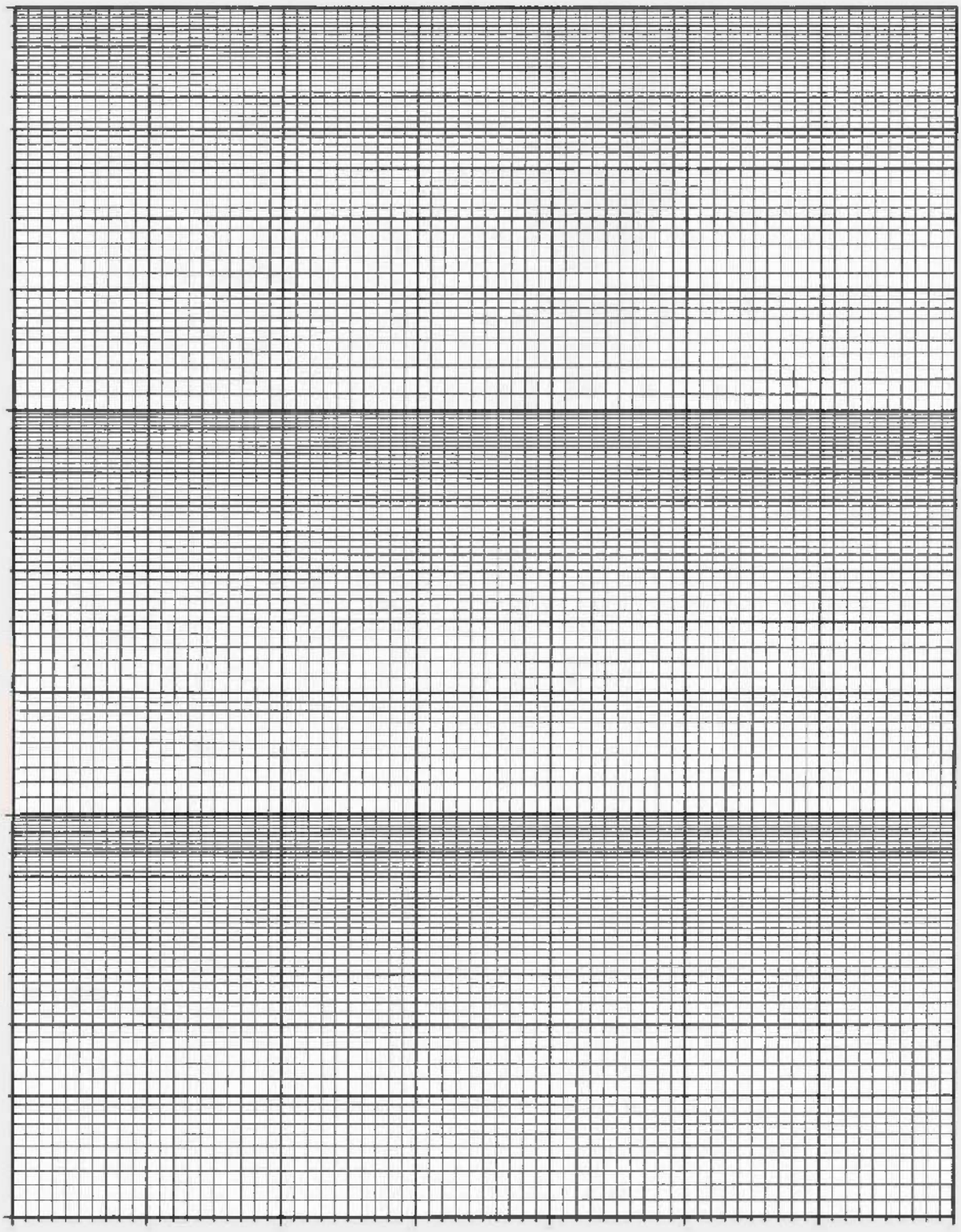
DATA SHEET 10-2

(continued)

5 *Why were the restriction enzymes kept on ice until they were used? Be specific in your answer.*

6 *Why are the enzymes the last ingredient added to the reaction mixture?*

7 *Examine Table 1. Why are the same volumes used in each reaction mixture?*



10

Bacterial Transformation: the pGLO™ System

EXERCISE 10-3

Theory

This exercise utilizes a kit produced by Bio-Rad Laboratories[1] that efficiently illustrates the following principles of microbial genetics:

- bacterial transformation
- use of an antibiotic selective medium to identify transformed cells
- the operon as a mechanism of microbial genetic regulation

Bacterial **transformation** is the process by which **competent** bacterial cells pick up DNA from the environment and make use of the genes it carries. It was first demonstrated in a strain of pneumococcus in 1928 by English bacteriologist Fred Griffith and since has been found to occur naturally in only certain genera. Modern techniques, however, have allowed biologists to make most cells (including eukaryotic cells) artificially competent, and this has made transformation a useful tool in genetic engineering. You will be using a $CaCl_2$ transforming solution and heat shock to make your *Escherichia coli* cells competent.

Green fluorescent protein (GFP) is responsible for **bioluminescence** in the jellyfish *Aequorea victoria* (Fig. 10.8). The GFP gene was isolated and altered so the GFP in this experiment fluoresces more than the natural version. You will be introducing the GFP gene into competent *E. coli* cells and will be using the ability of the cell to fluoresce as visual evidence of successful transformation and subsequent gene expression.

Operons are structural and functional genetic units of prokaryotes. Each operon minimally includes a **promoter site** (for binding **RNA polymerase**) and two or more **structural genes** coding for enzymes in the same metabolic pathway. In this exercise you will be using part of the arabinose operon.

The complete arabinose operon (Fig. 10.9) consists of the promoter (P_{BAD}) and three structural genes (*ara*B, *ara*A, and *ara*D) that code for enzymes used in arabinose digestion. In vivo, the arabinose enzymes are needed only when arabinose is present. After all, there is no point in the cell expending a lot of energy making the enzymes if the substrate is not there.

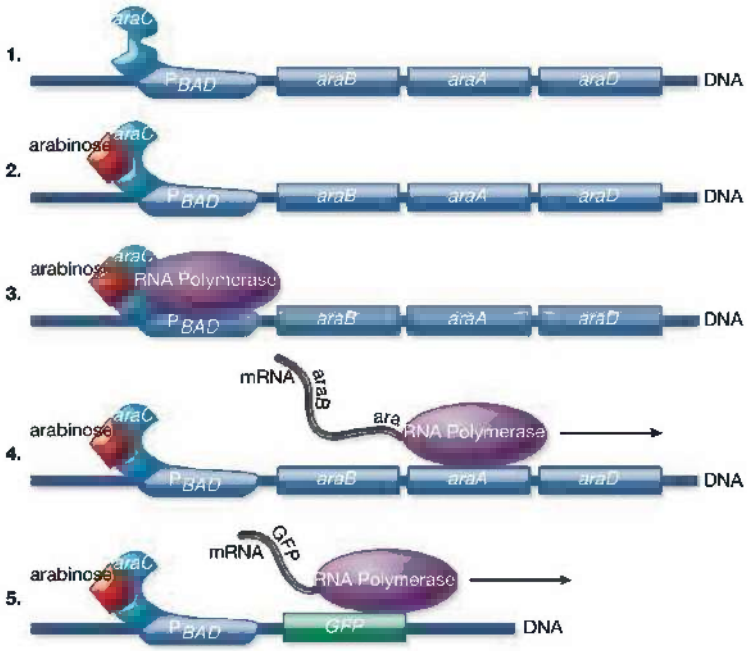
A DNA binding protein called *ara*C attaches to the promoter of the arabinose operon and acts like a switch. When arabinose is not present, *ara*C prevents RNA polymerase from binding to the promoter, so transcription *cannot* occur—the switch is "off." When arabinose is present, it binds to *ara*C and changes its shape so RNA polymerase *can* bind to the promoter and transcribe the genes—the switch is "on." Then this sequence of events occurs: The genes are transcribed, the enzymes are synthesized, they catalyze their reactions, and eventually the arabinose is consumed. Now, without arabinose *ara*C returns to its "off" shape and the genes are no longer transcribed. In this exercise you will be using the regulatory portion of the arabinose operon (the arabinose promoter and the *ara*C gene), but the structural genes have been replaced with the GFP gene.

All that remains is a means of carrying the GFP gene into the cell and replicating it—a **vector**. In this exercise you will be using a **plasmid** as a vector: the pGLO™ plasmid (Fig. 10.10). Plasmids are small, naturally occurring, circular DNA molecules that possess only a few genes and replicate independent of the chromosome (because they have their own replication origin). Although they are nonessential, they often carry genes that are beneficial to the bacterium, such as antibiotic resistance. The antibiotic resistance gene (*bla*) used in this experiment produces an enzyme called β-lactamase, which hydrolyzes certain antibiotics, including penicillin and ampicillin.

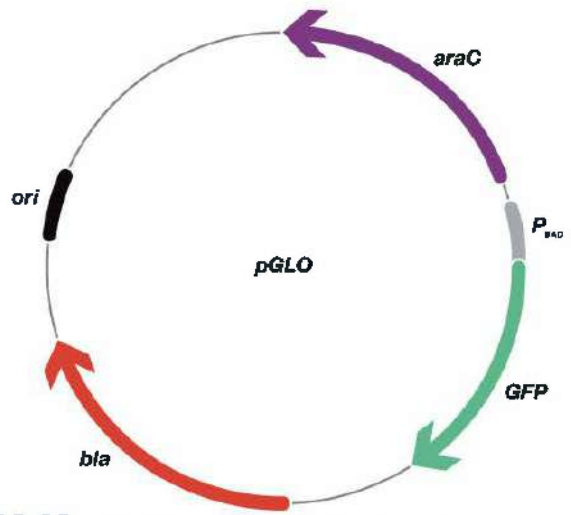
[1] Catalog Number 166-0003-EDU. Bio-Rad Laboratories Main Office 2000 Alfred Nobel Drive, Hercules, CA 94547; www.bio-rad.com, 1-800-424-6723.

10.8 Jellyfish *Aequorea victoria* ◆ (A) *A. victoria* produces GFP, but it is not visible to our eyes when illuminated with "natural" light. (B) When viewed with UV light, the ring of GFP around the edge of *A. victoria's* bell fluoresces.

(Photos taken at Cabrillo Marine Aquarium, San Pedro, CA.)



10.9 Functioning Arabinose Operon—Normal and Genetically Engineered ♦ The operons are prokaryotic structural and functional genetic units: They carry genes for enzymes in the same metabolic pathway, and they are regulated together. Operons with genes for catabolic enzymes are transcribed only when the specific substrate is present. In this case, the substrate is the pentose sugar arabinose. (1) The arabinose operon consists of a promoter (P_{BAD}) and three structural genes (*ara*B, *ara*A, and *ara*D). The DNA binding protein (*ara*C) attaches to the promoter and acts like a switch. Without arabinose present, RNA polymerase is unable to bind to the promoter and begin transcription of the genes. (2) and (3) Arabinose binds to a receptor on *ara*C and causes it to change to a shape that allows binding of RNA Polymerase to the promoter. (4) Transcription of the structural genes occurs, the enzymes are produced, and arabinose is catabolized. (5) The pGLO™ plasmid has been engineered to carry the arabinose promoter and the gene for green fluorescent protein (GFP) instead of the genes for arabinose catabolism. If arabinose is present, the GFP gene will be transcribed.



10.10 pGLO™ Plasmid ♦ The pGLO™ plasmid has been genetically engineered to carry a replication origin (*ori*), a bacterial promoter from the arabinose operon (P_{BAD}), the GFP gene (*GFP*) from the jellyfish *Aequorea victoria*, an antibiotic resistance gene (*bla*) coding for the enzyme β-lactamase, and the gene for the DNA binding protein from the arabinose operon (*araC*). Arrows indicate the direction of transcription.

The bottom line is this: The pGLO™ plasmid used in this experiment has been genetically engineered to contain the arabinose promoter (P_{BAD}), the gene for *ara*C, an antibiotic-resistance gene (*bla*), the gene for green fluorescent protein (GFP), and a replication origin (*ori*).

As you interpret the results of your experiment, you will see how these components come together and provide you with information about what is happening at the molecular level in your *E. coli* culture. Their functions are summarized in Table 10-2.

Application

Introduction of foreign DNA into a cell, identification of transformed cells, and regulation of expression of the introduced genes are skills used in genetic engineering.

In This Exercise

You will first make *E. coli* cells competent. Then, you will transform the competent *E. coli* cells with a plasmid containing the gene for green fluorescent protein. This

TABLE 10-2 Cast of Characters and a Legend of Abbreviations

Name	Symbol	Function in This Experiment
Green fluorescent protein	GFP	Used as an indicator of successful transformation and gene transcription (expression) in this experiment.
Plasmid	pGLO™	Used as a vector to introduce the GFP gene into recipient *E. coli* cells.
Arabinose promoter	P_{BAD}	Any promoter is the attachment site for RNA polymerase during transcription. *E. coli* RNA polymerase requires a prokaryotic promoter in order to transcribe genes, so the eukaryotic GFP must be spliced in next to one on the pGLO™ plasmid. (Normally, it is the attachment site for RNA polymerase during transcription of the *ara*B, *ara*A, and *ara*D genes.)
DNA binding protein	*ara*C	A regulatory molecule for the arabinose promoter. In the presence of arabinose, *ara*C has a shape that allows RNA polymerase to bind to the promoter. Without arabinose, *ara*C's shape prevents RNA polymerase from binding to the promoter.
Antibiotic resistance gene	*bla*	Produces β-lactamase, an enzyme that hydrolyzes certain antibiotics, including ampicillin. The gene provides a means of differentiating cells that were transformed and those that were not.
Replication origin	*ori*	Necessary for DNA replication. The pGLO™ plasmid is capable of replicating inside the cell because it has this. Because of replication, copies of the plasmid can be distributed to the descendants of the original, transformed *E. coli* cell.

technique of introducing a plasmid into cells is done routinely in genetic engineering protocols. You also will use arabinose as a genetic switch to regulate expression of the GFP gene by *E. coli*.

✓ Materials

Per Student Group

One Bio-Rad pGLO™ Transformation kit contains enough material for eight student workstations. Each workstation requires the following:

- ☐ One Luria-Bertani (LB) agar plate with *E. coli* colonies
- ☐ One sterile LB plate
- ☐ Two sterile LB + ampicillin plates (LB/amp)
- ☐ One sterile LB + ampicillin + arabinose plate (LB/amp/ara)
- ☐ One microtube of $CaCl_2$ transformation solution
- ☐ Two sterile microtubes
- ☐ One microtube of LB broth
- ☐ Seven disposable inoculating loops
- ☐ Five disposable calibrated transfer pipettes
- ☐ One foam microtube holder/float
- ☐ One container of crushed ice (big enough to accommodate the foam microtube holder)
- ☐ One marking pen

Per Class

In addition, a class supply of the following is required:

- ☐ Hydrated pGLO™ plasmid
- ☐ 42°C water bath and thermometer
- ☐ Long-wave UV lamp

Procedure

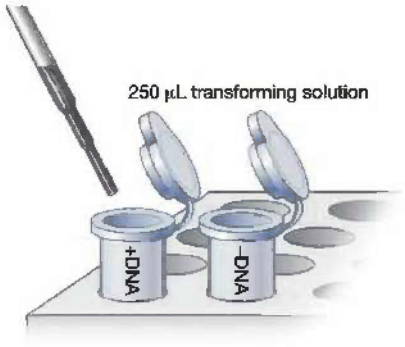
Lab One

Refer to the procedural diagram in Figure 10.11 as you read and perform the following procedure.

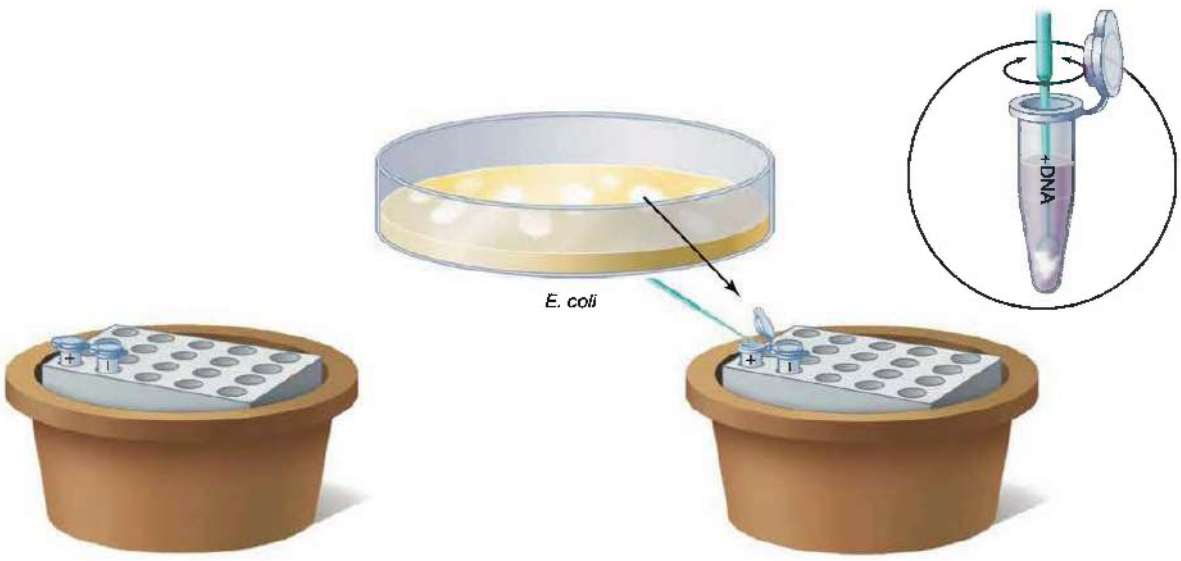
1. Obtain two closed microtubes. Label them with your group's name, then label one "+DNA" and the other "−DNA." Put both tubes in the microtube holder/float.
2. Using a sterile calibrated transfer pipette, dispense 250 µl of $CaCl_2$ transformation solution into each tube. Close the caps. The pipette calibration marks are shown in Figures 10.11 and 10.12.
3. Return the two tubes to the microtube holder/float and place them in the ice bath.
4. With a sterile loop, transfer one entire *E. coli* colony into the +DNA tube. Agitate the loop until all the growth is off of it and the cells are dispersed uniformly in the transformation solution. Close the lid and properly dispose of the loop.
5. Repeat step 4 with a sterile loop and the −DNA tube.
6. Hold the UV light next to the vial of pGLO™ plasmid solution. Record your observation on the data sheet, page 711.
7. Using a sterile loop, remove a loopful of pGLO™ plasmid DNA solution. Be sure there is a film across the loop. Then transfer the loopful of plasmid solution to the +DNA tube and mix.

1. Label one microtube "+DNA" and the other "–DNA."

2. Place the tubes in the foam microtube rack.



3. Use a sterile calibrated transfer pipette to dispense 250 µL transformation solution into each tube. Close the caps.



4. Place the microtube rack on the ice bath. Push the tubes down into the ice.

5. Transfer one *E. coli* colony to each tube using a different sterile loop. Agitate the loop to remove all growth (see detail). Close the caps and properly dispose of the loops.

10.11 Procedural Diagram ♦ Be sure to dispose of all pipettes and loops properly. *(Continues on next page.)*

10

8 Leave the tubes on ice. Make sure they are far enough down in the microtube holder/float that they make good contact with the ice. Leave them on ice for 10 minutes.

9 As the tubes are cooling on ice for 10 minutes, label the four LB agar plates.

- Label one LB/amp plate: +DNA
- Label the LB/amp/ara plate: +DNA
- Label the other LB/amp plate: −DNA
- Label the LB plate: −DNA

10 After 10 minutes on ice, transfer the microtube holder/float (with both tubes in it) to the 42°C water bath for exactly 50 seconds. This transfer from ice to warm water must be done rapidly. Also, make sure the tubes make good contact with the water.

11 After 50 seconds, quickly place both tubes back in the ice for 2 minutes. This process of heat shock makes the cell membranes more permeable to DNA. (Timing is critical. According to the kit's manufacturer, 50 seconds is optimal. No heat shock results in a 90% *reduction* in transformants,

6. Transfer one loopful of pGLO™ plasmid DNA to the "+DNA" tube only. Continue icing both tubes for 10 minutes.

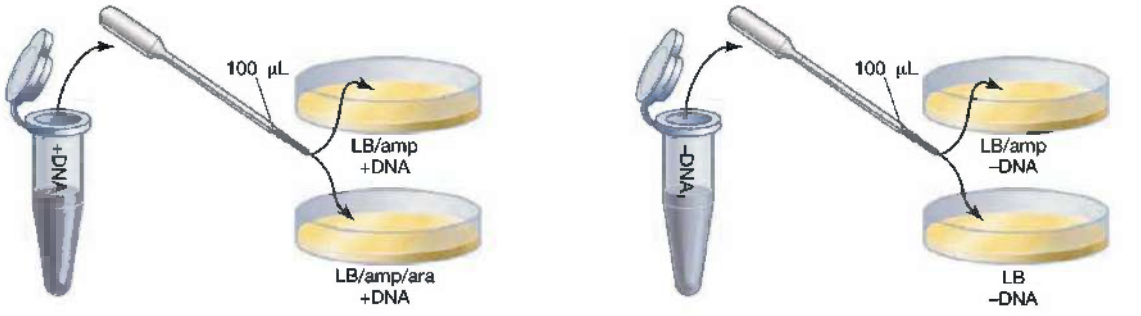
7. Quickly transfer the entire microtube rack to the 42°C water bath for exactly 50 seconds. Make sure the tubes contact the water.

8. Quickly place the microtube rack back on ice for two minutes.

9. Place the microtube rack on the table.

10. Add 250 µL LB broth to each tube with a different sterile transfer pipette.

11. Incubate the tubes for 10 minutes at room temperature.



12. Using a different pipette for each, transfer 100 µL of +DNA to an LB/amp and an LB/amp/ara plate. Using a different sterile loop for each, spread the inoculum for confluent growth. Use the "face" of the loop for this, not its edge. Incubate for 24 hours at 37°C.

13. Using a different pipette for each, transfer 100 µL of –DNA to an LB/amp and an LB plate. Using a different sterile loop for each, spread the inoculum for confluent growth. Use the "face" of the loop for this, not its edge. Incubate for 24 hours at 37°C.

10.11 Procedural Diagram *(Continued)* ♦ Be sure to dispose of all pipettes and loops properly.

whereas a 90-second heat shock yields about half the transformants.)

12 After 2 minutes, remove the microtube holder/float from the ice and place it on the table.

13 Using a sterile calibrated transfer pipette, add 250 µl of LB broth to the +DNA tube. Repeat with another sterile pipette and the –DNA tube. Properly dispose of both pipettes according to your lab's procedures.

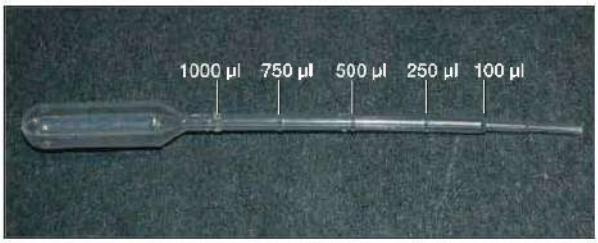
14 Incubate both tubes for 10 minutes at room temperature. Then mix the tubes by tapping them with your fingers.

15 Using a different sterile calibrated transfer pipette for each, inoculate the LB/amp/+DNA plate and the LB/amp/ara/+DNA plate with 100 µl from the +DNA tube.

16 Using a different sterile calibrated transfer pipette for each, inoculate the LB/amp/–DNA plate and the LB/–DNA plate with 100 µl from the –DNA tube.

17 Using a different sterile loop for each, spread the inoculum over the surface of all four plates to get confluent growth. Use the "face" of the loop for this, not its edge. Properly dispose of the loops.

18 Tape the plates together in a stack so they face the same direction. Then label them with your group name and the date, and incubate them for 24 hours at 37°C in an inverted position.



10.12 Disposable Transfer Pipette Calibrations The transfer pipettes supplied with the pGLO™ Transformation kit are calibrated to deliver specific volumes. The volumes and their corresponding lines are shown here.

Lab Two

1 Retrieve your plates. Observe them in ambient room light, then in the dark with UV illumination. Record your observations on the data sheet, and answer the questions.

2 When finished, properly dispose of all plates according to your lab's procedures.

Reference

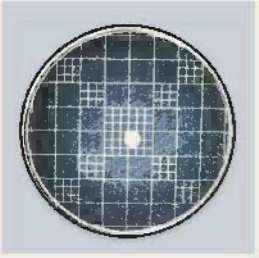
Bio-Rad Laboratories. Instruction pamphlet for the *Bacterial Transformation—The pGLO™ System* kit (Catalog Number 166- 0003-EDU). Bio-Rad Laboratories, Hercules, CA.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 10-3

Bacterial Transformation: the pGLO™ System

OBSERVATIONS AND INTERPRETATIONS

1 Record the appearance (clear, violet, or fluorescent green) of the pGLO™ plasmid solution with and without UV illumination.

2 In the following table, record your observations of the plates after incubation.

Plate	Inoculum	Number of Colonies	Appearance in Ambient Light	Appearance with UV Light
LB	*E. coli* (−pGLO™ DNA)			
LB/amp	*E. coli* (−pGLO™ DNA)			
LB/amp	*E. coli* (+pGLO™ DNA)			
LB/amp/ara	*E. coli* (+pGLO™ DNA)			

QUESTIONS

1 *In the following table, fill in the genotype of* E. coli *prior to transformation and after transformation.*

E. coli	GFP Gene (GFP$^+$ or GFP$^-$)	*Bla* (*Bla*$^+$ or *Bla*$^-$)
Before transformation		
After transformation		

2 *What was the purpose of examining the original pGLO™ solution with and without UV illumination?*

3 *What was the purpose of transferring the +DNA and −DNA tubes from ice to hot water to ice again?*

4 *Why were microtubes incubated for 10 minutes in LB broth rather than transferring their contents directly to the plates?*

5 *What information is provided by the LB/ – DNA plate?*

6 *Obviously, transformation could occur only if the pGLO™ plasmid was introduced into the solution. Which plates exhibit transformation?*

7 *What information is provided by the LB/amp/ – DNA plate?*

8 *Why does the LB/amp/ara/ + DNA plate fluoresce when the LB/amp/ + DNA plate does not?*

9 *Use the following information to calculate transformation efficiency.*

a. *You put 10 µL (one loopful) of a 0.03 µg/µL pGLO™ solution into the + DNA tube. Calculate the µg of DNA you used.*

b. *The + DNA tube contained 510 µL of solution prior to plating, but you did not use all of it. Calculate the fraction of the + DNA solution you used on each plate. Round off your answer to one decimal place.*

c. *Use your answers to questions 9a and 9b to calculate the micrograms of DNA you plated.*

10

d. *Using the number of colonies on the LB/amp/ara + DNA plate, calculate the transformation efficiency.* (**Hint:** *The units are transformants/µg of pGLO™ DNA.*)

e. *According to the manufacturer's (Bio-Rad Laboratories) manual, this protocol should yield a transformation efficiency between 8.0 × 10^2 and 7.0 × 10^3 transformed cells per microgram of pGLO™ DNA. How does your transformation efficiency compare? Account for any discrepancy.*

EXERCISE 10-4

Polymerase Chain Reaction

Theory

The polymerase chain reaction (PCR) is a technique conceived by the 1983 Chemistry Nobel Laureate Kary Mullis. The process is designed to make multiple copies (amplify) of a desired gene or other short DNA fragment. In the process, the double-stranded DNA (dsDNA) sequence to be amplified (the **template**) is separated and then replicated using free nucleotides, two commercially synthesized single-stranded DNA (ssDNA) **primers**, and a DNA replicating enzyme called a DNA polymerase. During the replication process, the double-stranded template DNA is separated into single strands, allowing the primers to anneal to their complementary target sequences. DNA polymerase then attaches to the 3' ends of the primers, which serve as nucleation sites (starting points), and uses the free nucleotides to synthesize new complementary DNA strands along the lengths of the template sequences. This process is outlined in Figure 10.13.

Primer design is critical to the success of PCR. The primer sequences are selected to be complementary to positions on opposite strands of the template DNA molecule, flanking the specific stretch of DNA to be replicated. They must be unique to one position on each template DNA to ensure binding at the desired location, that is, close to the DNA sequence to be replicated. Primers are typically 18 to 30 bases long to ensure this binding specificity. It is also important that they will not self-anneal (base pair with each other) or have the tendency to produce secondary structures, such as hairpins (structures produced when ends of the primer base pair, thus folding the primer in half).

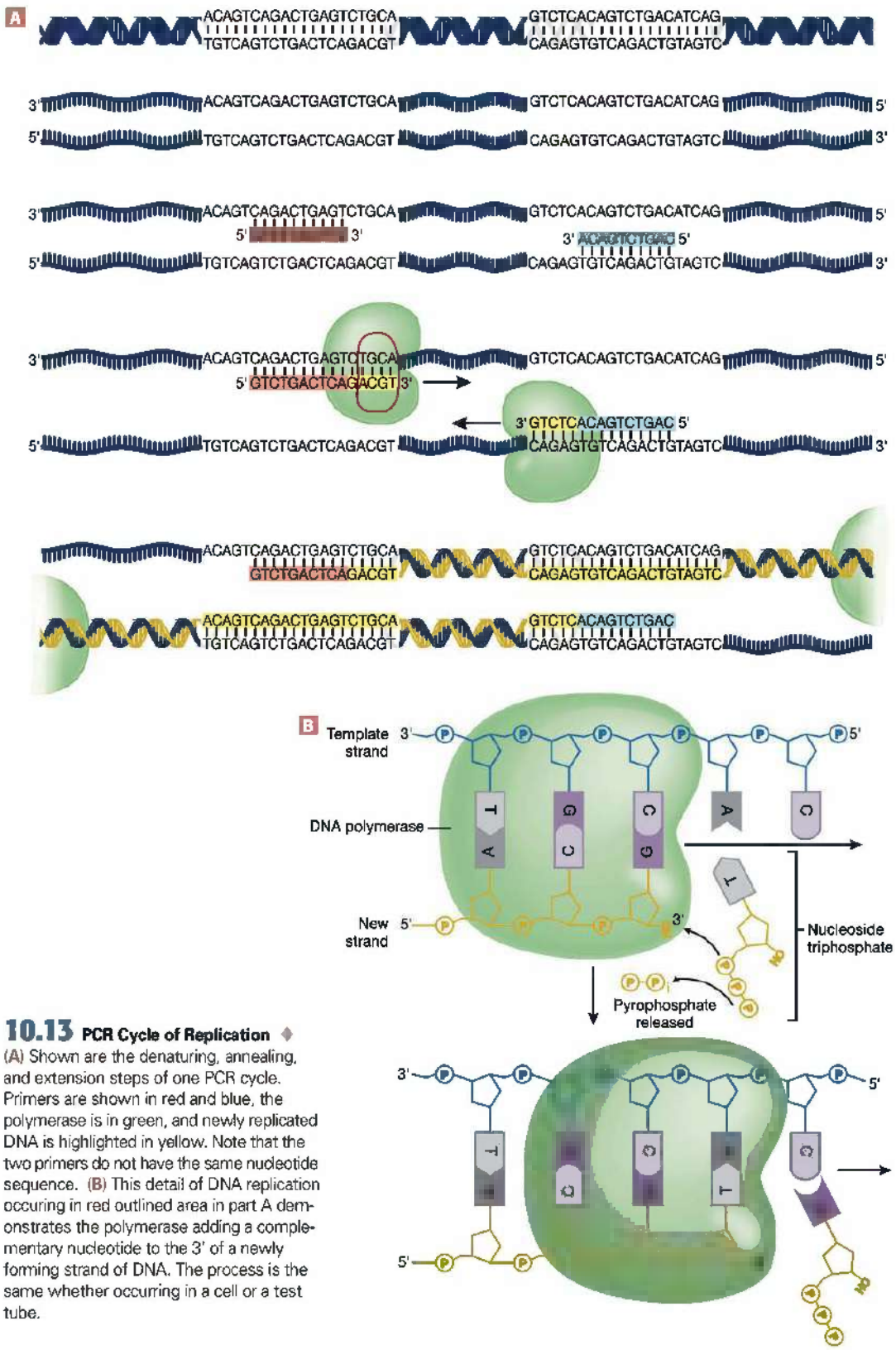
PCR involves 20 to 30 replication cycles (rounds of replication) of three different activities performed in sequence: denaturation (DNA strand separation), annealing (of primers), and extension (DNA replication). In a living cell (in vivo), these activities would be supported by a series of stabilizing enzymes. In a tube (in vitro), these activities are driven by temperature changes. In the denaturation step, the reaction tube's temperature is raised to 92°C–96°C for a few seconds to a few minutes to break the hydrogen bonds holding the double-stranded template DNA backbones together. The amount of time is determined based on the plastic tube's thickness and the efficiency of the machine performing the heating. During the annealing step, the temperature is lowered to 45°C–65°C to allow the primers to complementary base pair (anneal) to their target sequences on opposite strands of the now single-stranded template DNA. The necessary temperature depends on the melting temperature (T_m) of the primers being used. T_m is the temperature at which one-half of a particular dsDNA molecule will dissociate and become ssDNA, which depends on the percent of G – C pairs in the primer[1] and the nucleotide length. A quick mathematical calculation to determine an annealing temperature is $4(G + C) + 2(A + T) = T_m$°C, however, the best temperature is determined empirically using the result of this equation as a starting point. Lastly, for the extension step the temperature is raised to 72°C for a few seconds to a few minutes to allow the polymerase to synthesize new DNA strands complementary to the template. The amount of time depends on the base length of the expected product. As a general rule, 60 seconds per kilobase (kb) of DNA being synthesized is a good starting point for the protocol. 72°C is the optimal temperature for the commonly used enzyme *Taq* DNA polymerase to function.

These three steps complete the first cycle of amplification, or round of replication. The process then repeats itself (Figs. 10.13 and 10.14), but on the second round there are four single-stranded template molecules from the original two, and the product is eight strands of template DNA (four dsDNA molecules). By continuing the process and using the products of one cycle as templates for the next cycle, the number of DNA target strands doubles each cycle and can be amplified a millionfold, all in a few hours. (Assuming a single template at the start, 20 rounds of replication will produce 2^{20} = 1,048,576 double-stranded copies of the target DNA!)

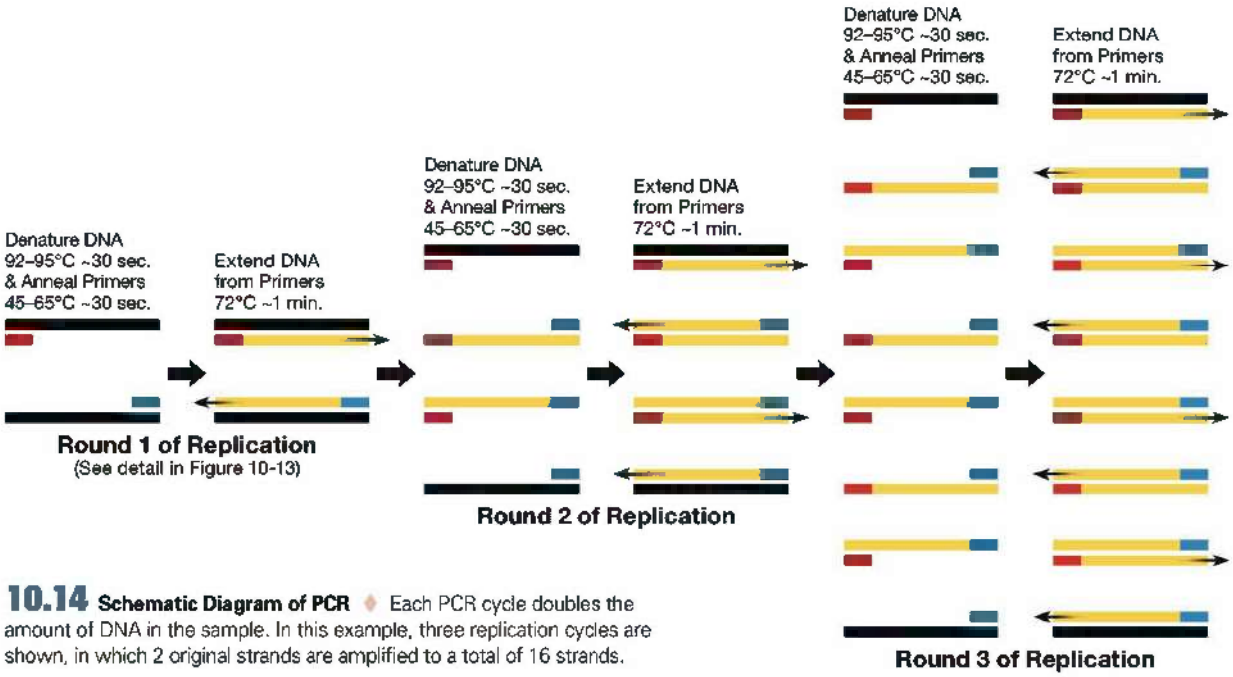
As mentioned above, two primers are used in the reaction, but they must added to the PCR reaction mixture in great excess to flood the mixture and promote primer-template annealing and discourage template-template reannealing. Other essential components of the mixture include sufficient quantities of free deoxyribonucleotides (dNTPs) in triphosphate form[2] (dATP, dCTP, dGTP, and dTTP) needed for DNA synthesis, buffers to maintain

[1] Because G-C pairs are held together by three hydrogen bonds and A-T pairs are held together by two, it requires more heat energy to separate a G-C pair. Therefore, the higher the G + C percent, the higher the T_m value.

[2] Being in triphosphate form allows them to supply the energy required for their insertion. dATP is the deoxyribose form of the familiar ATP (which has ribose as its sugar), but it has the same high energy bonds, as do the other dNTPs.



10.13 PCR Cycle of Replication ♦
(A) Shown are the denaturing, annealing, and extension steps of one PCR cycle. Primers are shown in red and blue, the polymerase is in green, and newly replicated DNA is highlighted in yellow. Note that the two primers do not have the same nucleotide sequence. (B) This detail of DNA replication occuring in red outlined area in part A demonstrates the polymerase adding a complementary nucleotide to the 3' of a newly forming strand of DNA. The process is the same whether occurring in a cell or a test tube.

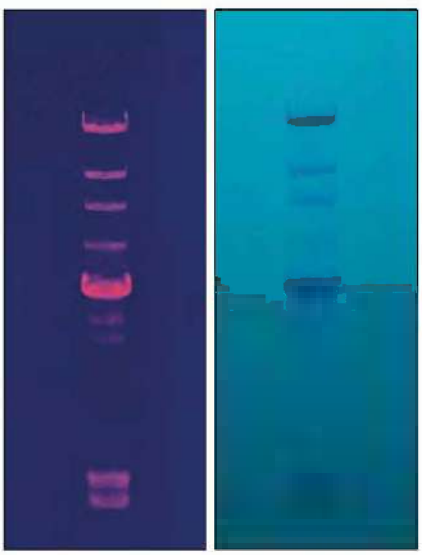


10.14 Schematic Diagram of PCR ♦ Each PCR cycle doubles the amount of DNA in the sample. In this example, three replication cycles are shown, in which 2 original strands are amplified to a total of 16 strands.

the appropriate pH, magnesium as a cofactor for the polymerase, and other salts to create an osmotic balance. Because of the high temperature required for the denaturation step, the thermotolerant *Taq* DNA polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, which occurs naturally in volcanic hot springs, is used to catalyze the extension.

Although Kary Mullis' original protocol involved moving the samples between water baths to achieve the temperature changes at each step, researchers now use a machine called a thermal cycler that can be programmed to rapidly perform these temperature shifts. A typical PCR thermal cycler program begins with a 5-minute hot start step at 95°C to ensure adequate mixing of all the reactants and to untangle the template DNA. This is followed by 20–30 rounds of replication involving the denaturation, annealing, and extension steps described previously. A single final extension step at 72°C for 5–10 minutes allows the DNA polymerase to extend any of the DNA pieces that might not have fully completed extension during the extension step of each round. The program typically ends with a hold step at 4°C to preserve the resulting DNA sample until the researcher is ready to use it.

Since DNA is tiny and colorless, the resulting PCR products cannot be visualized in the reaction tube. Agarose gel electrophoresis is frequently used in conjunction with PCR to separate DNA fragments of different sizes and allow visualization as bands in the gel. Many variables relating to shape, size, and a tendency of some fragments to form attachments to other molecules can affect migration patterns of DNA fragments. Generally speaking however, shorter DNA fragments will migrate faster and farther than longer fragments (Fig. 10.15 and illustrated on the data sheet, page 723). Simply looking at the bands on the gel will not tell you the fragment



10.15 Two Stained Gels ♦ The gel on the left was stained with ethidium bromide (EtBr) and viewed with UV light. The gel on the right was stained with methylene blue. Both gels show bands formed by the same standard DNA fragments. Your PCR reactions will each produce a single band.

sizes (lengths), but running a control sample of known DNA standards in the gel along with your samples allows construction of a standard curve that can be used to determine unknown fragment sizes. Agarose gel electrophoresis is explained more fully in Appendix G.

Application

The polymerase chain reaction (PCR) is a relatively simple and convenient method of amplifying DNA. Once multiple copies of the DNA are made, they can be used in various ways, ranging from research to diagnostics and forensics.

In This Exercise

You will be programming a thermal cycler, preparing a PCR reaction mixture, and running PCR. When your PCR is completed, you will run your samples, along with DNA standards (molecular weight marker controls), in agarose gel electrophoresis, and use your band migration distance to determine the resulting PCR DNA fragment sizes for comparison to your predictions from the pUC19 cloning vector plasmid map (Fig. 10.16).

Materials

For PCR Reactions:

Per Class

- ☐ Thermal cycler

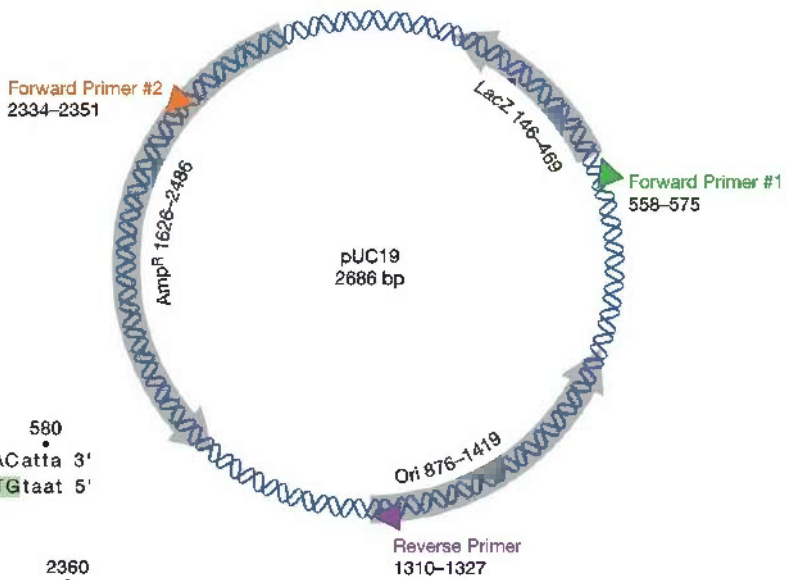
Per Student Group

- ☐ Microcentrifuge
- ☐ Pipettes and sterile tips to accommodate volumes from 1 µL to 200 µL
- ☐ Tip disposal container
- ☐ Microtube rack
- ☐ Six 0.5 mL thin wall PCR tubes
- ☐ Two 1.5 mL reaction tubes
- ☐ pUC19 template DNA (1 ng/µL)
- ☐ Forward primer #1 (50 µM)
- ☐ Forward primer #2 (50 µM)
- ☐ Reverse primer (50 µM)
- ☐ dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
- ☐ Standard *Taq* reaction buffer with 15 mM $MgCl_2$ (10×)
- ☐ *Taq* DNA polymerase on ice (5 units/µL)
- ☐ Enzyme grade ultrapure water
- ☐ Sterile mineral oil (for thermal cyclers without heated lids)

For Agarose Gel Electrophoresis (See Appendix G):

Per Class

- ☐ 50°C large water bath



10

Forward Primer #1 (558-575)

```
   550        560        570        580
5' gggtgccta ATGAGTGAGCTAACTCACatta 3'
3' cccacggatTACTCACTCGATTGAGTGtaat 5'
```

Forward Primer #2 (2334-2351)

```
  2330       2340       2350       2360
5' ttacc GCTGTTGAGATCCAGTTCgatgtaaccc 3'
3' aatggCGACAACTCTAGGTCAAGctacattggg 5'
```

Reverse Primer (1310-1327)

```
  1300       1315       1320       1330
5' cactagaagaa                    ctct 3'
3' gtgatcttctt GTCATAAACCATAGACGGgaga 5'
```

10.16 pUC19 Plasmid DNA Cloning Vector ◆ This diagram depicts a map of the 2686 base pair DNA plasmid called pUC19 that is serving as the template DNA in this exercise. The sequences and locations of the primers being used are shown. Numbers indicate the nucleotide base position on the plasmid.

Per Student Group

- ☐ Gel casting tray, two rubber dams or tape, and with an eight-well comb (wells should accommodate 60 µL each)
- ☐ Electrophoresis running chamber
- ☐ 1× TAE (Appendix H)
- ☐ Powdered agarose
- ☐ Balance and weigh paper
- ☐ 250 mL flask with foil cover
- ☐ Magnetic stir bar
- ☐ Magnetic hot plate
- ☐ 6× DNA loading dye (Appendix H)
- ☐ DNA standards (a molecular weight marker that can identify bands between 0.4 kb and 3 kb; 2-Log or 1 kb DNA Ladder is suggested)
- ☐ Micropipettors and sterile tips to accommodate volumes from 10 µL to 60 µL
- ☐ Electrophoresis power supply
- ☐ 0.1× TAE (Appendix H)
- ☐ DNA staining solution (Appendix H)

Procedure

Preparation for PCR (~30 min)

Follow the procedural diagram in Figure 10.17.

1. Program your thermal cycler for 30 cycles with the conditions shown below. Your instructor will show you how this is done.

   Step 1: 95°C, 5 min. (hot start)

   Step 2: 95°C, 35 sec. (denature)

   Step 3: 55°C, 40 sec. (annealing)

   Step 4: 72°C, 2 min. (extension)

   Step 5: repeat steps 2–4, 29 times

   Step 6: 72°C, 10 min. (final extension)

   Step 7: 4°C, indefinitely (hold)

2. Using the table on the data sheet, page 721, calculate the necessary quantities of each reagent to prepare your master mixes for the two different PCR reactions you will be doing. A master mix is made when you are repeating multiple reactions of the same reagents. It allows you to save time and tips when pipetting. (*Note*: Your instructor may assign you only one set of primers for one reaction master mix, depending on resources available. You should still complete all the calculations in the table in step 1 on the data sheet.)

3. Put on latex or nitrile gloves. Obtain your reagents and set up the master mix reactions according to your plan, using the two 1.5 mL tubes that you have labeled Rxn #1 and Rxn #2. Keep in mind that the volumes being used are very small and care must be taken to pipette accurately (Appendix D). Use a clean pipette tip between each transfer and watch that the full quantity of each reagent gets delivered to the master mix tube by dispensing the reagent against the inside surface of the tube. Do not be concerned with everything getting to the bottom of the tube while you are setting up your reactions. Keep the *Taq* DNA polymerase on ice at all times and add this to your reactions last. When adding the *Taq* DNA polymerase, pipette up and down gently a few times in the reaction mixture to ensure delivery, because the enzyme solution is thick and tends to stick to the pipette tip.

4. Cap the reaction tubes tightly and tap or vortex them GENTLY to mix the ingredients.

5. Place the tubes in a microcentrifuge for 20 seconds to force the mixture to the bottom of the tube. Be sure to balance the centrifuge before starting the spin cycle.

6. Label three of the 0.5 mL PCR tubes for Rxn #1 and the three time points you will be collecting: 10, 20, and 30 cycles/rounds of replication. Dispense 50 µL of Rxn #1 master mix into each of the three tubes, cap them tightly and keep the PCR tubes on ice. Discard the remaining master mix.

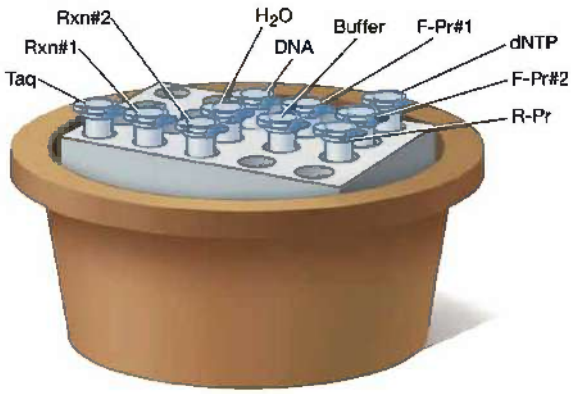
7. Label the remaining three 0.5 mL PCR tubes for Rxn #2 and the three time points you will be collecting: 10, 20, and 30 cycles/rounds of replication. Dispense 50 µL of Rxn #2 master mix into each of the three tubes, cap them tightly and keep the PCR tubes on ice. Discard the remaining master mix.

8. Place the PCR tubes in a microcentrifuge for 20 seconds to force the mixture to the bottom of the tube. Be sure to balance the centrifuge before starting the spin cycle.

10

PCR (~2 hours)

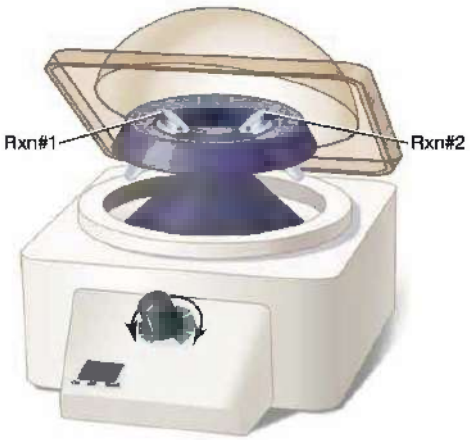
1. If your thermal cycler has a heated lid, place your six PCR tubes inside as shown by your instructor. If your thermal cycler does not have a heated lid, add a drop of mineral oil to each tube before placing the tubes in the machine. Secure the lid and press start to begin the program.



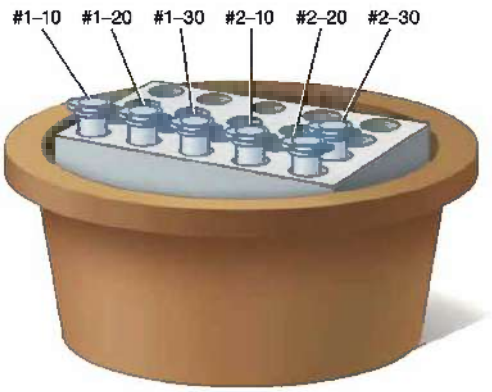
1. Gather your reagents and label two 1.5 mL tubes as shown.

2. Pipette reagents to create your two master mixes.

3. Mix by tapping or vortexing gently.



4. Centrifuge for 20 seconds.



5. Label six 0.5 mL PCR tubes as shown.

6. Pipette 50 µL of the appropriate master mix into each PCR tube.

10

10.17 PCR Procedural Diagram

(Continued on next page.)

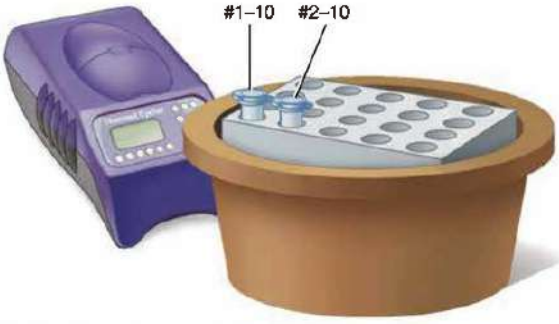
2 While you are waiting for the PCR program to run, prepare your agarose gel according to the instructions in Appendix G, Module A. Once the gel has been poured, work on calculating the sizes of PCR DNA fragments that are expected with each set of primers, using the information provided in the map of pUC19 template DNA in Figure 10-16. Record your predictions on the data sheet, page 721.

3 After completing 10 cycles (approximately 37 minutes into the program), remove the 10-cycle PCR tube for both reactions and place them on ice.

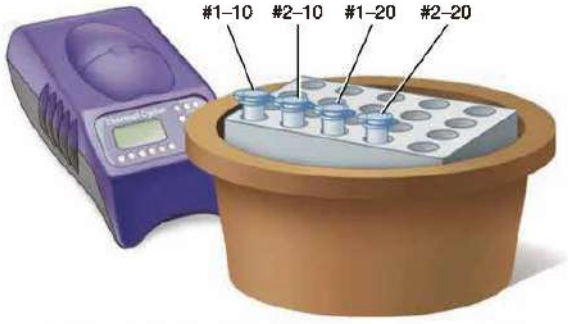
4 After completing 20 cycles (approximately 70 minutes into the program), remove the 20-cycle PCR tube for both reactions and place them on ice.

7. Centrifuge for 20 seconds.

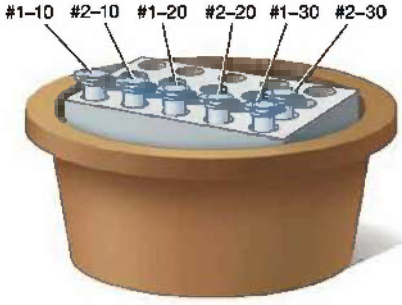
8. Place the PCR tubes in the thermal cycler, close the lid, and start.



9. After 10 cycles, open the thermal cycler and remove the "10" tubes and place on ice. Close the lid immediately.



10. After 20 cycles, open the thermal cycler and remove the "20" tubes and place on ice. Close the lid immediately.



11. When the program is complete, remove the last set of tubes and place on ice.

10.17 PCR Procedural Diagram *(Continued)* ♦

10

5 When the PCR program is complete (approximately 2 hours), remove the 30-cycle PCR tubes and place them on ice. Proceed to the next step, or store the six PCR reaction tubes and your gel in the refrigerator until the next lab period.

Agarose Gel Electrophoresis (20 minutes to 2 hours, depending on gel size and voltage used).

1 Using a different pipette tip for each transfer, add 10 µL of 6X DNA loading dye to each PCR reaction tube.

2 Cap the reaction tubes tightly and tap or vortex them GENTLY to mix the ingredients.

3. Place the tubes in a microcentrifuge for 20 seconds to force the mixture to the bottom of the tube. Be sure to balance the centrifuge before starting the spin cycle.
4. Record the well position of each sample in the gel and load all 60 µL of each reaction tube.
5. Load 5 µg of a DNA Ladder molecular weight marker in loading dye in an empty well adjacent to your samples. This sample will serve as controls for the experiment.
6. Run your gel according to the instructions in Appendix G, Module B.

Gel Staining and Destaining (4 hours to overnight)

1. Stain and destain the agarose gel according to the instructions in Appendix G, Module C.
2. Compare your results to the PCR fragment sizes you predicted from the pUC19 map. Measure the migration distance of your two different PCR products and complete the table and graph on the data sheet.

References

Krebs, Jocelyn E., Elliott S. Goldstein, and Stephen T. Kilpatrick. Section 3.8 in *Lewin's GENES XI*, 11th ed. Burlington, MA: Jones and Bartlett Learning, 2012.

Madigan, Michael T., John M. Martinko, David Stahl. Chap. 11 in *Brock Biology of Microorganisms*, 13th ed. Boston: Benjamin Cummings, 2010.

Nelson, David L. and Michael M. Cox. Chaps. 8 and 9 in *Lehninger Principles of Biochemistry*, 6th ed. New York: W.H. Freeman, 2012.

New England BioLabs. "PCR Protocol for *Taq* DNA Polymerase with Standard *Taq* Buffer (M0273)." Available online. URL: https://www.neb.com/protocols/1/01/01/taq-dna-polymerase-with-standard-taq-buffer-m0273

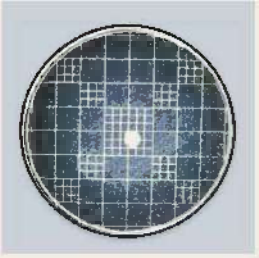
Watson, James D., Richard M. Meyers, and Amy A. Caudy. Chap. 6 in *Recombinant DNA: Genes and Genomes–A Short Course*, 3rd ed. Long Island, NY: Cold Spring Harbor Laboratory Press, 2007.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 10-4

Polymerase Chain Reaction

OBSERVATIONS AND INTERPRETATIONS

1 Use the following table to plan the master mixes for your PCR reactions.

Table 1 PCR Reaction Planner

Stock Reagent	Final Concentration of Reagent	Amount Needed to Make Final Concentration in 50 µL Reaction	Master Mix for 4 PCR Reactions of Forward Primer #1 & Reverse Primer (Rxn #1)	Master Mix for 4 PCR Reactions of Forward Primer #2 & Reverse Primer (Rxn #2)
Water	To 50 µL			
10× Buffer	1×			
10 mM dNTPs	0.2 mM	1 µL	4 µL	
1 ng/µL pUC19 template DNA	1 ng			
50 µM Forward Primer #1	0.5 µM			–
50 µM Forward Primer #2	0.5µM		–	
50 µM Reverse Primer	0.5µM			
5 units/µL Taq	1.25 units			
Total Volume	**50 µL**	**50 µL**	**200 µL**	**200 µL**

Example calculation: dNTPS are supplied at a stock concentration of 10 mM (C_1) and are used at a final concentration of 0.2 mM (C_2) in the 50 µL reaction (V_2). Solve for Volume 1 (V_1), the amount of dNTP stock you will need for the reaction.

$$C_1V_1 = C_2V_2$$
$$10\ \text{mM} \times V_1 = 0.2\ \text{mM} \times 50\ \mu\text{L}$$
$$V_1 = (0.2\ \text{mM} \times 50\ \mu\text{L}) \div 10\ \text{mM}$$
$$V_1 = 1\ \mu\text{L}$$

As calculated where 1 µL of the stock dNTPs is used In a 50 µL PCR reaction. For the master mix, multiply this by 4. *Note:* To reach a total PCR reaction volume of 50 µL, calculate the water volume by subtracting the sum of the other reagents from 50 µL. Always add the *Taq* DNA polymerase after all the other reagents have been added.

2 Predict your results. Use the pUC19 plasmid cloning vector map in Figure 10.16 to calculate the expected fragment sizes that will be generated when the template DNA is amplified using the provided primer sets.

Forward Primer #1 and Reverse Primer (Rxn #1):

Forward Primer #2 and Reverse Primer (Rxn #2):

3 Following gel electrophoresis, measure the migration distances for some of the DNA standard fragments and enter your data in the right-hand column in the following table. For consistency, position the gel well-side at the top and measure from the bottom of the well to the bottom of each band. It is best not to include the largest or smallest fragments but do include at least five, and up to 10, fragments that span a range of sizes. For example, if you are using the 2-log DNA Ladder shown in the illustration (step 6) on page 723, the 0.5 kb, 0.8 kb, 1.0 kb, 1.5 kb, 2.0 kb, 3.0 kb, 4.0 kb and 6.0 kb fragments are likely easily visible and should produce good data for generating your standard curve.

Table 2 **Migration Distances (cm) and Fragment Size (kb)**

Reaction #1 (30 cycle band)		Reaction #2 (30 cycle band)		DNA standards	
Distance (cm)	Size (kb)	Distance (cm)	Size (kb)	Distance (cm)	Size (kb)

10

4 Measure the migration distance of the band in your 30-cycle lane from your two different PCR reactions in the same manner as described in step 3. Enter the data in the left-hand side of each column in Table 2.

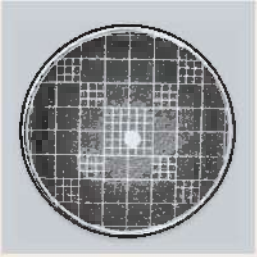
5 Using the migration distance data in Table 2 and the base pair length data of the DNA standards you are using, construct a standard curve on the semilogarithmic graph paper provided. Do not connect the data points; draw a straight trend line through them as centrally located as possible. (For an example see Exercise 10-2, Data Sheet, page 701, Standard Curve.)

Name _____

Date _____

Lab Section _____

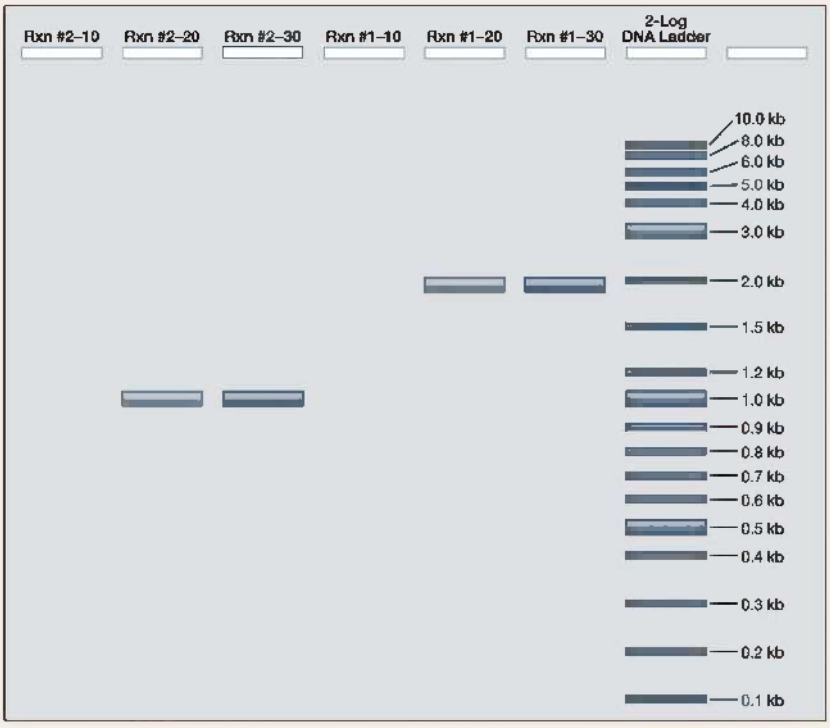
I was present and performed this exercise (initials) _____



DATA SHEET 10-4

(continued)

6 Use the standard curve and the data in Table 2 to plot the migration distances of your PCR fragments on the semilogarithmic graph paper provided on page 726. To do this, locate the migration distance on the x-axis and draw a straight vertical line from that point up to the standard curve. Then draw a horizontal line from that point of intersection with the standard curve to the y-axis. The place where the line intersects the y-axis is the estimated number of base pairs in the DNA fragment. Enter the data in the right-hand side of each column in Table 2 and compare these results with the sizes that you predicted in step 2.



Gel Schematic ♦ This is an idealized drawing of a PCR agarose gel. The base pair sizes indicated are those of the DNA standard called 2-Log DNA Ladder that was suggested for use in this experiment. Consult with your instructor for the sizes of the DNA standards used in your experiment.

QUESTIONS

1 *Did your observed PCR fragment sizes match your predicted fragment sizes as calculated from the pUC19 plasmid map? If not, what factors do you think might have contributed to a discrepancy?*

2 *In each reaction mixture, which PCR band of the three time points contained the most DNA? Why did you conclude this?*

3 *Why is* Taq *DNA polymerase, rather than a polymerase from a mesophile, like* Escherichia coli *for example, used for the extension step?*

4 *Genomes are frequently described according to their G + C content. Would you expect to program a thermal cycler the same way for an organism with a 50% G + C as an organism with 25% G + C? If yes, why? If not, what would you change?*

10

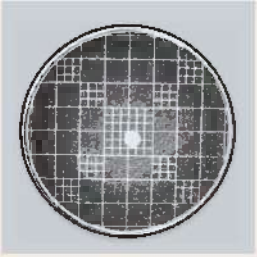
5 *Why do you think it is necessary to wear gloves when setting up PCR reactions?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

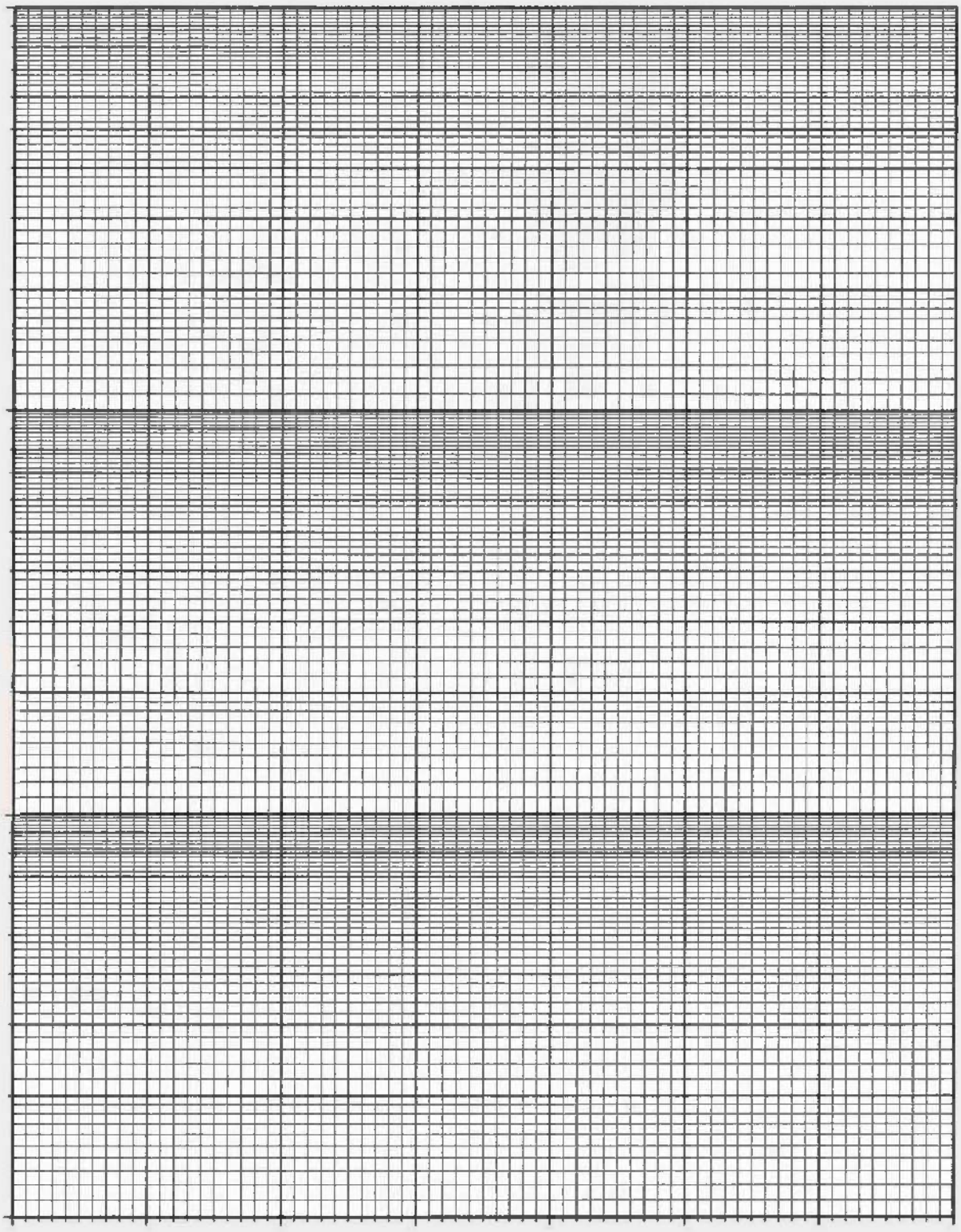


DATA SHEET 10-4

(continued)

6 *Why was the* Taq *DNA polymerase kept on ice until it was used? Be specific in your answer.*

7 *Why is* Taq *DNA polymerase the last ingredient added to the reaction mixture?*



Ultraviolet Radiation Damage and Repair

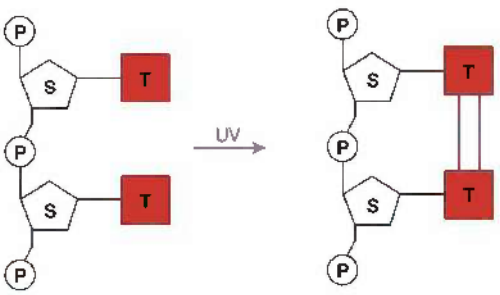
EXERCISE 10-5

Theory

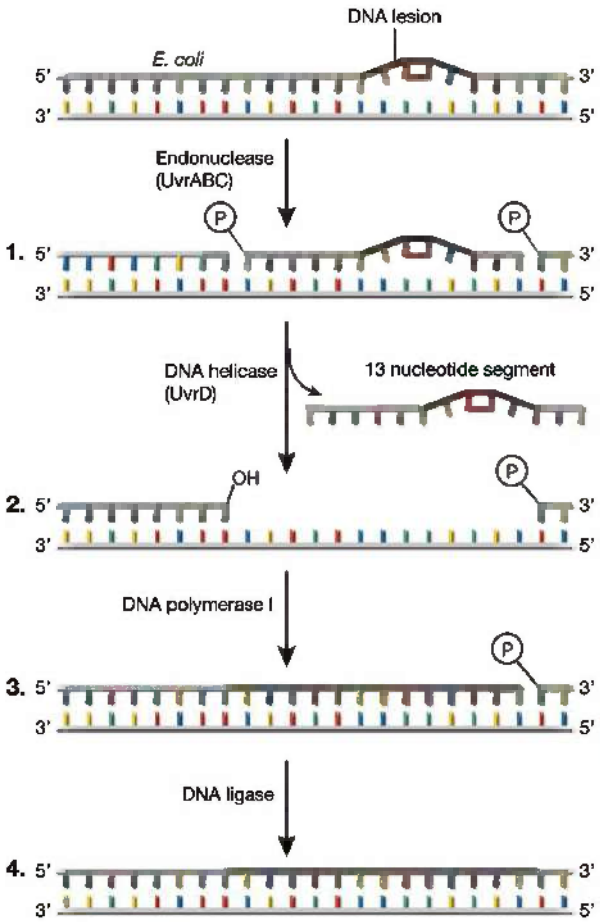
Ultraviolet radiation is part of the electromagnetic spectrum, but with shorter, higher energy wavelengths than visible light. Prolonged exposure can be lethal to cells because when DNA absorbs UV radiation at 254 nm, the energy is used to form new covalent bonds between adjacent pyrimidines: cytosine-cytosine, cytosine-thymine, or thymine-thymine. Collectively, these are known as **pyrimidine dimers**, with thymine dimers being the most common. These dimers distort the DNA molecule and interfere with DNA replication and transcription (Fig. 10.18).

Many bacteria have mechanisms to repair such DNA damage. *Escherichia coli* performs **light repair**, or **photoreactivation**, in which the repair enzyme, **DNA photolyase**, is activated by visible light (300–500 nm) and simply monomerizes the dimer by reversing the original reaction.

A second *E. coli* repair mechanism, **excision repair**, or **dark repair**, involves a number of enzymes (Fig. 10.19). The thymine dimer distorts the sugar-phosphate backbone of the strand. This is detected by UvrABC **endonuclease** (also known as ABC exinuclease) that breaks two bonds—one eight nucleotides in the 5' direction from the dimer, and the other four nucleotides in the 3' direction. A **helicase** (UvrD, also known as helicase II) removes the 14-nucleotide fragment (including the dimer), leaving single-stranded DNA. **DNA polymerase I** inserts the appropriate complementary nucleotides in a 5' to 3' direction to make the molecule double-stranded again. Finally, **DNA ligase** closes the gap between the last nucleotide of the new segment and the first nucleotide of the old DNA, and the repair is complete. Both mechanisms are capable of repairing a small amount of damage, but long and/or intense exposures to UV produce more damage than the cell can repair, making UV radiation lethal.



10.18 Photoreactivation Repair of a Thymine Dimer in One DNA Strand ♦ Thymine (or any pyrimidine) dimers form when DNA absorbs UV radiation with wavelengths in the neighborhood of 260 nm. The energy is used to form two new covalent bonds between adjacent thymines, resulting in distortion of the DNA strand. In photoreactivation repair, DNA photolyase can break this bond to return the DNA strand to its normal shape and function. If it doesn't, and excision repair fails, the distortion interferes with DNA replication and transcription.



10.19 Excision, or Dark Repair, in *E. coli* ♦ Four enzymes are used in this repair process: (1) An endonuclease (UvrABC) to break two covalent bonds in the sugar-phosphate backbone of the damaged strand, (2) a helicase (UvrD) to remove the nucleotides in the damaged segment, (3) DNA polymerase I to synthesize a new strand, and (4) DNA ligase to form a covalent bond between the new and the original strands.

10

Application

Because ultraviolet radiation has a lethal effect on bacterial cells, it can be used in decontamination. Its use is limited, however, because it penetrates materials such as glass and plastic, poorly. In addition, bacterial cells have natural mechanisms to repair UV damage.

In This Exercise

The lethal effects of ultraviolet radiation, its ability to penetrate various objects, and the cells' ability to repair UV damage will all be demonstrated. Do not be misled by the apparent simplicity of the experimental design—there is a lot going on!

✓ Materials

Per Student Group

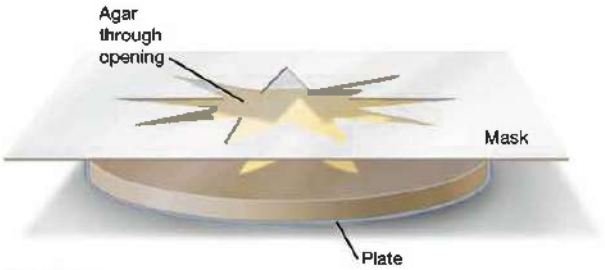
- □ Seven nutrient agar plates
- □ (Optional) three TGYM plates
- □ Disinfectant
- □ Poster-board masks with 1" to 2" cutouts (Fig. 10.20)
- □ Short wavelength ultraviolet lamp (UV-C) with appropriate shielding and support
- □ Sterile cotton applicators
- □ 24-hour tryptic soy broth culture of *Serratia marcescens*
- □ (Optional) 24-hour TGYM culture of *Deinococcus radiodurans* or *D. radiophilus*

Procedure

Lab One

Refer to the procedural diagram in Figure 10.21 as you read and follow this procedure.[1]

1. Using a sterile cotton applicator, streak an agar plate to form a bacterial lawn over the entire surface by using a tight pattern of streaks. Rotate the plate one-third of a turn and repeat, then rotate it another one-third of a turn and streak one last time. Repeat this process for all remaining plates.
2. Number the plates 1, 2, 3, 4, 5, 6, and 7.
3. Remove the lid from plate 1 and set it on a disinfectant-soaked towel. Place the plate under the UV light and cover it with a mask.



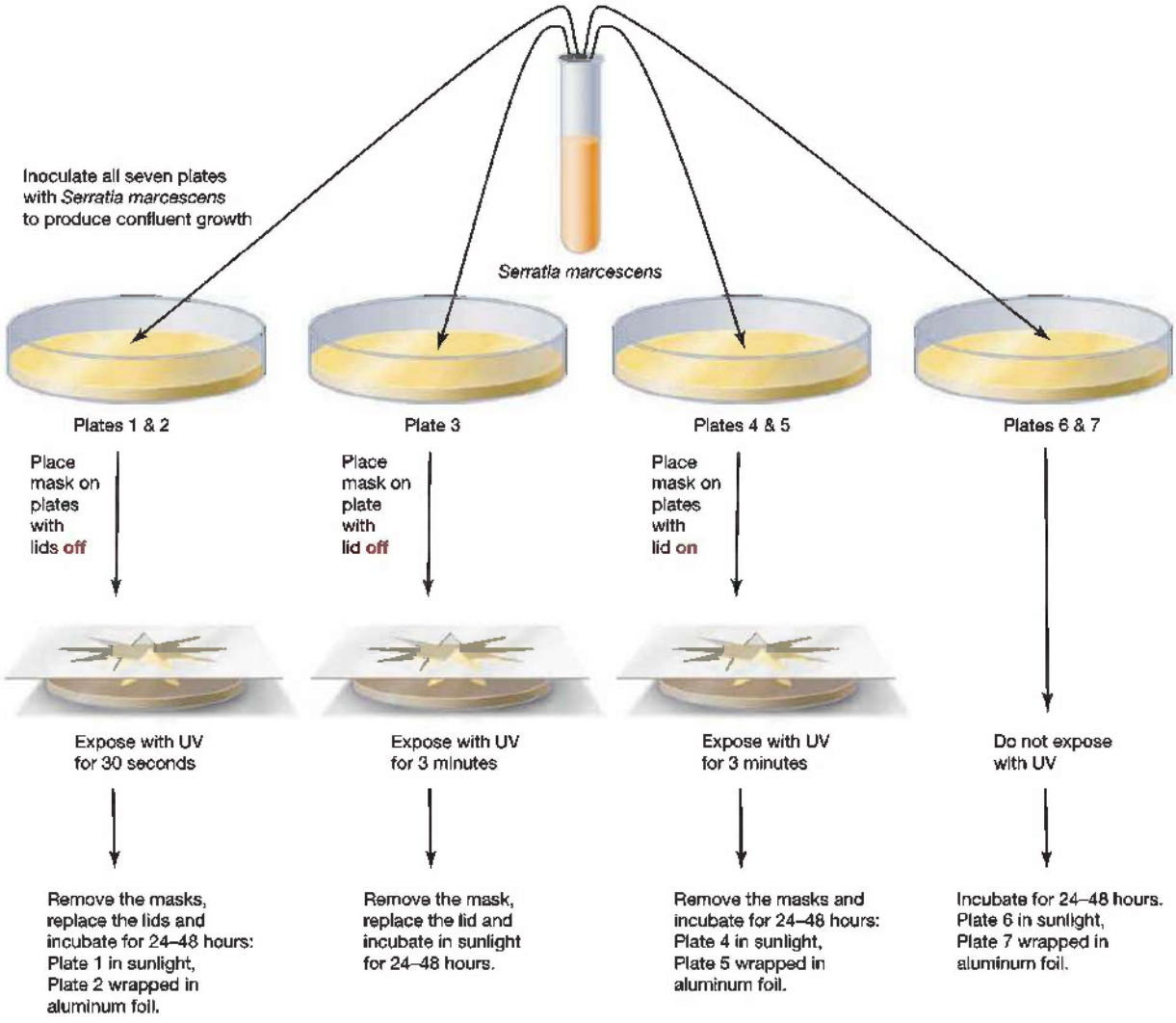
10.20 Poster-board Mask ◆ This is an example of a mask placed over a Petri dish. The cutout may be any shape but should leave the outer 25% of the plate masked.

4. Turn the UV lamp on, but do not look at it. After 30 seconds, turn off the UV lamp, remove the mask, and replace its lid. If space permits, you may combine this step with step 5.
5. Repeat the process for plate 2. If space permits, you may combine this step with plate 1 in step 4.
6. Repeat the process for plate 3, but leave the UV lamp on for 3 minutes. If space permits, you may combine this step with plates 4 and 5 in steps 7 and 8.
7. Irradiate plate 4 for 3 minutes, but leave the lid on and cover with the mask.
8. Repeat step 7 for plate 5.
9. Do not irradiate plates 6 and 7.
10. Incubate plates 1, 3, 4, and 6 for 24 to 48 hours at room temperature in an inverted position where they can receive natural light (e.g., a windowsill). Do not stack them. (Usually 24 hours is plenty of time. Longer incubation can lead to pigment loss by *S. marcescens*.
11. Wrap plates 2, 5, and 7 in aluminum foil, invert them, and place with the others for 24 to 48 hours. Do not stack them unless space is limited.
12. (Optional) Inoculate three TGYM plates with *Deinococcus* spp. as in step 1. Then, expose them to 5, 10, and 20 minutes of UV irradiation with a mask but no lid. Label the plates 8, 9, and 10, and with their appropriate exposure times. Incubate them in sunlight for 24 to 48 hours with the other plates.

Lab Two

1. Remove the plates and examine the growth patterns.
2. Record your results on the data sheet, page 731.

[1] Thanks to Roberta Pettriess of Wichita State University for her helpful suggestions to improve this exercise.


Inoculate all seven plates with *Serratia marcescens* to produce confluent growth
Serratia marcescens
Plates 1 & 2
Plate 3
Plates 4 & 5
Plates 6 & 7
Place mask on plates with lids **off**
Place mask on plate with lid **off**
Place mask on plates with lid **on**
Expose with UV for 30 seconds
Expose with UV for 3 minutes
Expose with UV for 3 minutes
Do not expose with UV
Remove the masks, replace the lids and incubate for 24–48 hours: Plate 1 in sunlight, Plate 2 wrapped in aluminum foil.
Remove the mask, replace the lid and incubate in sunlight for 24–48 hours.
Remove the masks and incubate for 24–48 hours: Plate 4 in sunlight, Plate 5 wrapped in aluminum foil.
Incubate for 24–48 hours. Plate 6 in sunlight, Plate 7 wrapped in aluminum foil.

10.21 Procedural Diagram ◆ Inoculate and expose the plates as directed. Be sure to shield the UV light source adequately. Do not look at the light.

References

Alcorn, Joseph L. and Claud S. Rupert. *Journal of Bacteriology* 172, no. 12 (1990): 6885–6891.

Moat, Albert G., John W. Foster, and Michael P. Spector. Chap. 3 in *Microbial Physiology*, 4th ed. New York: Wiley-Liss, 2002.

Nelson, David L. and Michael M. Cox. Chap. 25 in *Lehninger's Principles of Biochemistry*. New York: W. H. Freeman and Co., 2005.

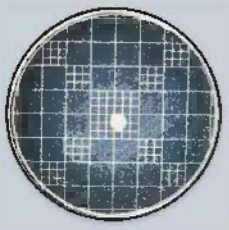
White, David. Chap.19 in *The Physiology and Biochemistry of Prokaryotes*. New York: Oxford University Press, 2000.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 10-5

Ultraviolet Radiation Damage and Repair

OBSERVATIONS AND INTERPRETATIONS

1 Describe the growth of *Serratia marcescens* in both parts of each plate (i.e., where the mask shielded the growth from UV and where it did not) in the following table. Use the terms *confluent, dense growth*; *confluent, sparse growth*; *individual colonies*; and *no growth* for your descriptions. Make a note as to the significance of each type of growth. Do the same for *Deinococcus* if you did the optional procedure (plates 8, 9 and 10).

Plate	Mask/Lid	UV Exposure Time (min.)	Incubation	Growth on Agar Surface Covered by Mask	Growth on Agar Surface Beneath Opening of Mask
1	Mask, no lid	0.5	Sunlight		
2	Mask, no lid	0.5	Dark		
3	Mask, no lid	3	Sunlight		
4	Mask and lid	3	Sunlight		
5	Mask and lid	3	Dark		
6	Lid only	0	Sunlight	NA	
7	Lid only	0	Dark	NA	
8	Mask, no lid	5	Sunlight		
9	Mask, no lid	10	Sunlight		
10	Mask, no lid	20	Sunlight		

QUESTIONS

1 *Consider the exposed part of the plate.*

a. *What does the relative sparseness of growth tell you about the effect of UV radiation?*

b. *What do individual colonies tell you about the cells from which they grew?*

2 *What does the relatively heavy growth on the plates covered by the mask and lid during exposure tell you about UV radiation?*

3 *What is the effect of longer UV exposure on* Serratia marcescens*? To answer this question, which plates should be compared? (Think carefully about your answer.)*

4 *What is the effect of the poster-board mask on UV radiation? To answer this question, which plates should be examined? (Think carefully about your answer.)*

5 *What is the effect of the plastic lid on UV radiation? To answer this question, which plates should be compared? (Think carefully about your answer.)*

10

6 *When you incubated the plates in sunlight, why could you be fairly certain that no further UV damage would be inflicted on the cells? (Think carefully about your answer.)*

EXERCISE
10-6

Ames Test

Theory

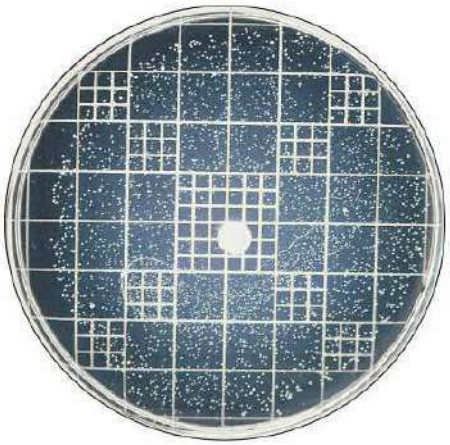
Bacteria that are able to enzymatically synthesize a particular necessary biochemical (such as an amino acid) are called **prototrophs** for that biochemical. If the bacterial strain can make the amino acid histidine, for instance, it is classified as a histidine prototroph. Bacteria that must be supplied with that biochemical (e.g., histidine) are called (histidine) **auxotrophs.** Auxotrophs typically are created from prototrophs when a mutation occurs in the gene coding for an enzyme used in the pathway of the biochemical's synthesis.

The Ames test employs mutant strains of *Salmonella typhimurium* that have lost their ability to synthesize histidine. One histidine auxotroph strain—TA1538—possesses a **frameshift mutation** in the histidine operon; that is, they are missing one or have an extra nucleotide in the DNA sequence that otherwise would code for an enzyme necessary for histidine production. Another strain—TA1535—possesses a **missense substitution mutation,** in which one nucleotide in the histidine gene has been replaced, resulting in a faulty gene product.

The Ames test determines the ability of chemical agents to cause a reversal—a **back mutation**—of these auxotrophs to the original prototrophic state. In this test, histidine auxotrophs are spread onto **minimal agar plates** that contain all nutrients for growth but only a trace of histidine.

When a filter paper disk saturated with a suspected mutagen is placed in the middle of the minimal agar plate, the substance will diffuse outward into the medium. If it is mutagenic, it will cause back mutation in some cells (converting them to histidine prototrophs) that freely grow into full-size colonies. (***Note***: A small amount of histidine is included in the medium to allow the auxotrophs to grow for several generations and expose them to the effects of the mutagen. The unmutated auxotrophs typically exhibit faint growth but do not develop into full-size colonies because of the rapid exhaustion of the histidine.) A sample plate is shown in Figure 10.22.

To make the test more sensitive to mutagens, the *Salmonella* strains used also have nonfunctional DNA repair mechanisms and lack the lipopolysaccharide layer found in Gram-negative cells. The former prevents cells from repairing any mutations caused by the chemical being tested. The latter makes it easier for the tested chemical to enter the cell.



10.22 Testing for Mutagenicity ♦ In this example, a *Salmonella* histidine auxotroph was grown on histidine minimal agar while exposed to a suspected mutagen. The colonies were derived from cells that underwent back mutations. It is not known from this plate alone, however, whether they are a result of the effects of the test substance or simply spontaneous mutations, so it must be compared to a control minimal agar plate in which a nonmutagenic substance (DMSO) is substituted for the test substance.

Several variations of the Ames test are possible. For instance, the test may be run with liver extract in the medium to mimic the human body's ability to convert some harmless chemicals into mutagenic products, but you will not be doing this. The Ames test protocol you will be doing uses two minimal agar plates and two **complete agar plates.** Complete agar contains all of the nutrients necessary for growth of *Salmonella*. All four plates are inoculated with histidine auxotrophs. One minimal agar plate and one complete agar plate each receive a filter paper disk saturated with the test substance. The second minimal agar plate and complete agar plate each receive a filter paper disk saturated with dimethyl sulfoxide, DMSO (a substance known to be nonmutagenic).

The minimal agar plate containing the test substance determines mutagenicity of the test substance. Only prototrophs will grow on minimal agar, so recovery of any colonies on the minimal agar inoculated with auxotrophs is indicative of back mutation and there is evidence that the chemical is a carcinogen. Depending on the strain used, the type of mutation (either frameshift or base substitution) also can be determined. The minimal

agar plate with DMSO serves as a control for the minimal agar/test substance plate by measuring **spontaneous back mutations** (natural mutations that occur without the presence of a mutagen).

The purpose of the complete agar plate containing the test substance is a control to evaluate toxicity of the test substance. Creation of a **zone of inhibition** around the disk indicates toxicity (Fig. 10.23). The more toxic the substance, the larger the zone will be. If the substance is toxic, there may be no indication of mutagenicity, because the cells are killed before they can back mutate. Finally, the complete agar plate containing DMSO serves as a control for comparison to the growth and zone of inhibition on the complete agar/test substance plate.

Application

Many substances that are mutagenic to bacteria are also carcinogenic to higher animals. The Ames test is a rapid, inexpensive means of using specific bacteria to evaluate the mutagenic properties of potential carcinogens. Many variations of the basic Ames test are used in specific applications.

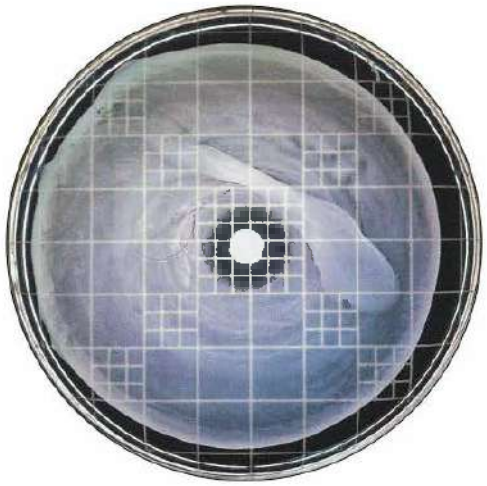
In This Exercise

You will test a household substance for its mutagenic properties. If you want the best chance of a positive result, check below your sink and in the garage for products that have many organic chemicals in the ingredient list. When handling these materials, be sure to wear gloves. Use caution in the laboratory if the material is flammable.

Materials

Per Student Group

- □ Four minimal medium (MM) plates
- □ Four complete medium (CM) plates (***Note:*** This exercise can be run with two plates of each medium if replicates are not desired.)
- □ Centrifuge
- □ Two sterile centrifuge tubes
- □ Small beaker containing alcohol and forceps
- □ Bottle of 1 × Vogel-Bonner salts (Appendix H)
- □ Eight sterile filter disks made with a paper punch
- □ Two sterile Petri dishes (for soaking filter paper disks)
- □ Two sterile transfer pipettes
- □ 100 µl–1,000 µl digital pipettors and tips
- □ Container for disposal of supernatant (to be autoclaved)
- □ DMSO



10.23 Testing for Toxicity ◆ Shown is a *Salmonella* histidine auxotroph grown on complete agar (containing histidine) and exposed to the test substance. The zone of inhibition around the paper disk indicates toxicity of the substance. (Note that a zone of inhibition is visible on the plate shown in Figure 10.22, but it is not as well defined as on complete agar because the growth is not as dense.)

- □ Test substance (any substance that has possible mutagenic properties and **does not contain histidine or protein**)
- □ Hand tally counter
- □ Broth culture[1] of *Salmonella typhimurium* TA1535
- □ Broth culture[1] of *Salmonella typhimurium* TA1538

Procedure

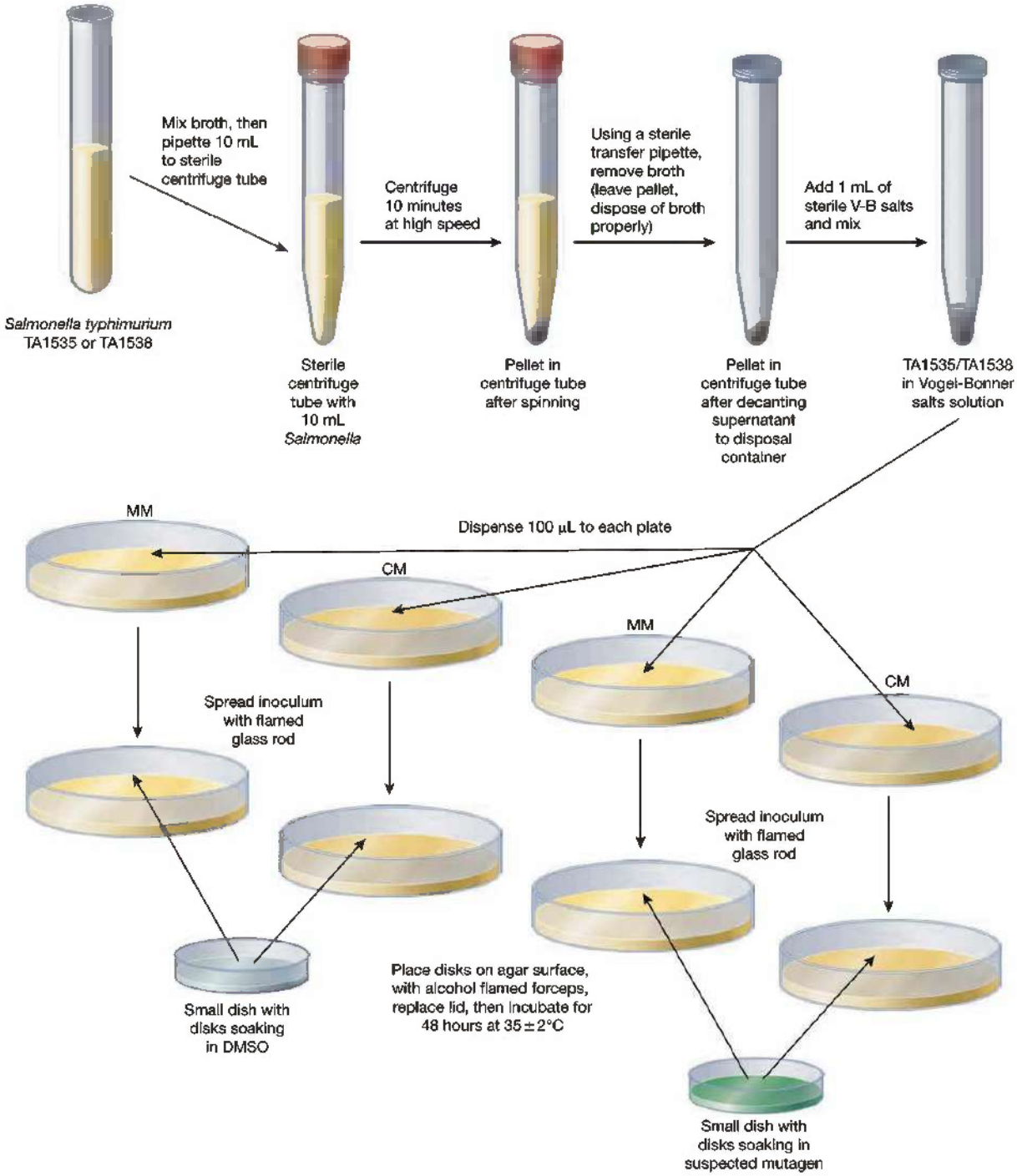
Lab One

Follow the procedural diagram in Figure 10.24.

1. Soak four filter paper disks in DMSO and four filter paper disks in the test substance.
2. Pipette 10.0 mL TA1535 into a sterile centrifuge tube. Do the same with TA1538, and then label the tubes.
3. Centrifuge the tubes on high speed for 10 minutes. Be sure the centrifuge is balanced.

[1] The cultures used for this exercise must be prepared as follows:

1. 24 hours before the test, inoculate two 10.0 mL broth tubes with TA1535 and TA1538. Incubate at 35 ± 2°C together with two sterile 90.0 mL broths (in a 100 mL diluent bottle).
2. 5½ hours before the test, pour the TA1538 culture into one of the sterile 90.0 mL broths and return it to the incubator until time for the exercise.
3. 4 hours before the test, pour the TA1535 culture into the other 90.0 mL sterile broth and return it to the incubator until time for the exercise.


Mix broth, then pipette 10 mL to sterile centrifuge tube
Centrifuge 10 minutes at high speed
Using a sterile transfer pipette, remove broth (leave pellet, dispose of broth properly)
Add 1 mL of sterile V-B salts and mix
Salmonella typhimurium TA1535 or TA1538
Sterile centrifuge tube with 10 mL Salmonella
Pellet in centrifuge tube after spinning
Pellet in centrifuge tube after decanting supernatant to disposal container
TA1535/TA1538 in Vogel-Bonner salts solution
Dispense 100 µL to each plate
MM
CM
MM
CM
Spread inoculum with flamed glass rod
Spread inoculum with flamed glass rod
Small dish with disks soaking in DMSO
Place disks on agar surface, with alcohol flamed forceps, replace lid, then incubate for 48 hours at 35 ± 2°C
Small dish with disks soaking in suspected mutagen

10.24 Procedural Diagram ◆ Be sure to dispose of all pipettes, broth, and tubes properly as you perform the experiment. After 48 hours of incubation, use a metric ruler to measure the diameter of the cleared zone on all CM plates. Use a colony counter to count the back-mutant colonies on all the MM plates. (***Note:*** These will be fairly large. Do not count the tiny "hazy" growth over the plate's surface.) Mark each colony with a toothpick (to avoid counting it more than once) as you keep track of the number using a hand tally counter.

4. Being careful not to disturb the cell pellet at the bottom, decant the supernatant (the fluid at the top) from each centrifuge tube using a different sterile transfer pipette.
5. Resuspend the cell pellets by adding 1.0 mL sterile 1× Vogel-Bonner salts to each tube and mixing well.
6. Using the spread plate technique (Exercise 1-5), inoculate two MM plates and two CM plates with 100 µl of the resuspended TA1535. Do the same with TA1538.
7. Flame the forceps by passing them through the Bunsen burner flame, and allowing the alcohol to burn off.
8. Using the flamed forceps, place the DMSO and test disks in the centers of the plates. Gently tap down the disks with the forceps to ensure good contact for diffusion of the test substance/DMSO and to prevent them falling off when the plates are inverted.
9. Incubate the plates aerobically at 35 ± 2°C for 48 hours.

Lab Two

1. Measure the diameters (in millimeters) of the zones of inhibition on the CM plates.
2. Count the colonies on the MM plates and compare. Count only the large colonies, not the "hazy" background growth. (These are the colonies produced by auxotrophs that did not back mutate to prototrophs and only grew until the histidine in the minimal medium was exhausted.)
3. Record your observations for the Ames test plates on the data sheet, page 737.

References

Ames, Bruce N., Frank D. Lee, and William E. Durston. "An Improved Bacterial Test System for the Detection and Classification of Mutagens and Carcinogens." *PNAS* 70, no. 3 (1973): 782–786.

Eisenstadt, Bruce, C. Carlton, and Barbara J. Brown. Page 311 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Maron, D. M. and B. N. Ames. *Mutation Research* 113 (1983): 173–215.

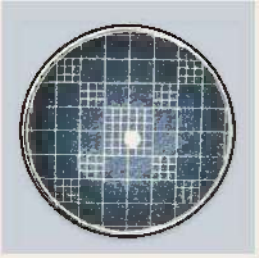
Nelson, David L. and Michael M. Cox. Chap. 25 in *Lehninger's Principles of Biochemistry*. New York: W. H. Freeman and Co., 2005.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 10-6

Ames Test

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations for the Ames test plates in the following table. On the CM plates, measure the zone of inhibition diameter in millimeters. On the MM plates, count only the large colonies, not the "hazy" background growth. (These are the colonies produced by auxotrophs that did not back mutate to prototrophs and grew only until the histidine in the minimal medium was exhausted.)

	Zone Diameter		Colonies Counted	
	CM with TA 1535	CM with TA 1538	MM with TA 1535	MM with TA 1538
DMSO				
Test substance				

QUESTIONS

1 *Which plates do you have to examine to determine if your test substance is toxic? What is your conclusion about your test substance?*

2 *Which plates do you need to examine to determine if your test substance is mutagenic? What is your conclusion about your test substance?*

3 *Why can't your test substance contain protein?*

4 *What is the purpose of including a small amount of histidine in minimal medium?*

5 *Why are the small colonies on the minimal medium plates not considered back mutants? Why don't they grow to full size?*

10

6 *What are some advantages of the Ames test over carcinogen tests using rats or mice? What are some disadvantages?*

Phage Typing of *E. Coli* Strains

EXERCISE 10-7

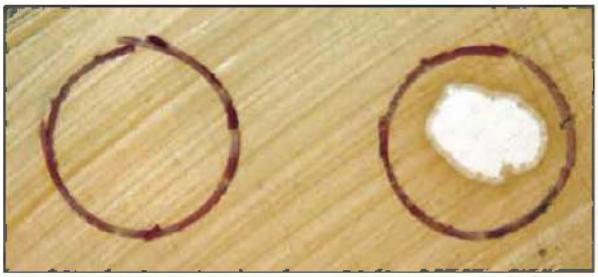
Theory

Identification of organisms is a primary function of microbiologists. The best identification methods involve utilizing genetically based differences to distinguish between organisms at every level: Domain through species. In microbiology classical identification methods include cell morphology, staining properties, biochemical testing, and serological reactions, all of which are still being used. Newer methods include comparisons of nucleic acids, such as hybridization and sequencing. **Phage typing** allows microbiologists to differentiate between strains of bacterial species based on susceptibility to infection by different viruses.

Viruses that infect bacteria are called **bacteriophages**, or simply just **phages**. The infection process (Fig. 6.13) begins with attachment by the phage to a receptor on the host cell. The phage then injects its nucleic acid into the host cytoplasm and overtakes its metabolism; the host is now under viral control and becomes a factory for making a hundred or more copies of the virus. When completed, the host cell bursts and releases the viral progeny, which now infect other cells to repeat the replicative cycle. Depending on the virus, one round of replication can take as little as 25 minutes at 37°C!

The ability of a virus to infect a host has to do with its ability to attach to *specific* host receptors. Because receptor structure is genetically determined, different genetic strains of the bacterial species can be identified by their susceptibility to a particular virus. Challenging the same host with different viruses (which use different receptors) produces a composite picture of its viral susceptibility. Different composite pictures indicate different *host* strains and provide a means of characterizing them based on their viral susceptibilities. This characterization can be used as a basis for identifying fresh isolates down to the level of "strain."

One way of determining host susceptibility to a particular phage is to inoculate a plate with the host so it produces confluent growth (a lawn). Phage can be introduced at the same time and the plate incubated for 24–48 hours. Clear zones in the lawn are evidence that the virus has gone through multiple replicative cycles and destroyed all the host cells in that region. If no clearing is seen, then the bacterial strain is not a suitable host for that virus (Fig. 10.25).



10.25 Zone of Clearing and No Clearing ◆ This plate was inoculated to produce a lawn of host bacteria. Two different viruses were spotted onto the plate, which was then incubated for 48 hours. Notice the clearing on the right and absence of clearing on the left. (For convenience, the places where virus was applied are circled.)

Application

Phage typing is used to characterize and identify strains within a bacterial species.

In This Exercise

Today you will be phage typing three strains of *Escherichia coli* (K-12, B, and C) by inoculating agar plates with each strain and with two *E. coli* bacteriophages (T_4 and $\phi\chi174$).

Materials

Per Student Group

- ☐ Overnight broth cultures of
  - *E. coli* K-12
  - *E. coli* B
  - *E. coli* C
- ☐ Three nutrient agar or tryptic soy agar plates
- ☐ Microtube with 250 µL of T_4 phage
- ☐ Microtube with 250 µL of $\phi\chi174$ phage
- ☐ Microtube with 250 µL of sterile distilled water
- ☐ 1.5 mL transfer pipettes
- ☐ Marking pen

10

Procedure

Lab One

1. With your marking pen, divide each agar plate into three sectors. Label one sector "T_4," a second sector "$\phi\chi174$," and the third sector "dH_2O" (Fig. 10.26).
2. Then, label one plate "*E. coli* K-12," a second plate "*E. coli* B," and the third plate "*E. coli* C."
3. Take the *E. coli* K-12 plate and, with a sterile swab, streak it with *E. coli* K-12 to produce a lawn. Your goal is confluent growth, so make the streaks right next to each other. When you have covered the surface, rotate the plate 1/3 turn and repeat the streaking of the inoculum already on the plate using the same technique to produce confluent growth. Then, rotate the plate another 1/3 turn and repeat. (As an alternative, the spread plate technique may be used.) ***Note:*** Failure to produce a lawn of confluent growth may mean your results are compromised. Take this inoculation process seriously.
4. Repeat Step 3 with the *E. coli* B plate and *E. coli* B culture.
5. Repeat Step 3 with the *E. coli* C plate and *E. coli* C culture.
6. With the small transfer pipette, transfer a *small* drop of T_4 phage to the appropriate sector on the *E. coli* K-12 plate (Fig. 10.27). DO NOT touch the agar with the pipette. Simply lower it until a drop contacts the agar, then lift the pipette. Properly dispose of the pipette.
7. Repeat Step 6 using phage $\phi\chi174$, on the *E. coli* K-12 plate.
8. Repeat Step 6 using sterile distilled water on the *E. coli* K-12 plate.
9. Repeat Steps 6 through 8 using the *E. coli* B plate.
10. Repeat Steps 6 through 8 using the *E. coli* C plate.
11. Allow the phage/water drops to soak into the agar for about 10 minutes.
12. Tape the plates and incubate them inverted at 35 ± 2°C for 24–48 hours.

Lab Two

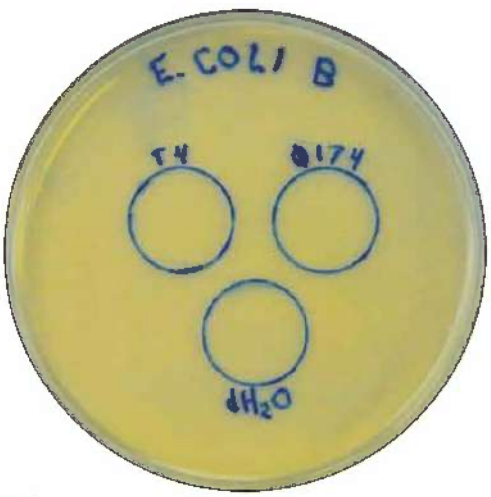
1. Examine the plates for zones of clearing where the phage/sterile water was applied.
2. Record your results and answer the questions on the data sheet, page 741.

References

Acheson, Nicholas H. *Fundamentals of Virology*. Hoboken, NJ: John Wiley and Sons, 2006.

Rees, Catherine E. D. and Martin J. Loessner. *Phage Identification of Bacteria*. Chap. 8 in *Practical Handbook of Microbiology*, 2nd. ed. Emanuel Goldman and Lorrence H. Green, eds. Boca Raton, FL: CRC Press, Taylor and Francis Group, 2009.

Shors, Teri. *Understanding Viruses*. Sudbury, MA: Jones and Bartlett Publishers, Inc., 2009.



10.26 Labeling the Plate ♦ On each plate's base, divide the agar into three sectors with your marking pen. Label the sectors "T_4," "$\phi\chi174$," and "dH_2O." Also label each plate with one of the *E. coli* strains.

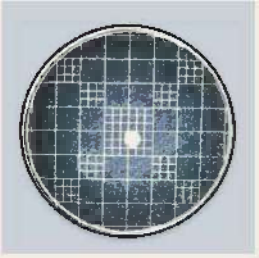
10.27 Applying the Phage ♦ Use a small transfer pipette to put a *small* drop of the appropriate phage/dH_2O on each plate sector. Do not touch the agar with the pipette or use it more than once.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 10-7

Phage Typing of *E. coli* Strains

OBSERVATIONS AND INTERPRETATIONS

1 Record your results in the following table. Use "+" for clearing and "–" for no clearing.

E. coli Strain	Sterile Water	Phage T_4	$\phi X174$
K-12			
B			
C			

QUESTIONS

1 *What is the purpose of phage typing?*

2 *What would a clear zone produced by the sterile water mean? How would that affect the interpretation of your results?*

3 *Suppose you were told these results for a third phage, T_7:*

E. coli Strain	Phage T_7
K-12	–
B	+
C	–

a. *Could you tell* E. coli *K-12 and* E. coli *C apart based exclusively on these results? Explain.*

10

b. *If your answer was "no," suggest what could be done to help you differentiate between them. Be thorough.*

SECTION 11

Hematology and Serology

This section deals with blood cells and practical applications of the immune system. In Exercise 11-1 you will have the opportunity to perform a differential blood cell count and observe many of the cells involved in the body's defense system. Exercises 11-2 through 11-6 allow you to perform serological tests that are used to detect the presence of antigens or antibodies in a sample. Exercises 11-2 and 11-3 introduce the basic concept and utility of antigen/antibody specificity, which is at the heart of all serological reactions. They also introduce the necessity of an "indicator reaction" to show that antigen and antibody have reacted. These use precipitation, a type of test that is rarely used any more, but is a comfortable way of introducing these concepts. Exercises 11-4 and 11-5 introduce serological tests in which the indicator is agglutination. Exercise 11-6 illustrates a modern technique, the ELISA test.

Differential Blood Cell Count

EXERCISE 11-1

Theory

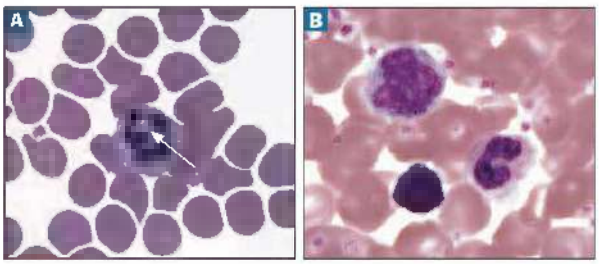
Leukocytes (white blood cells, or WBCs) are divided into two groups: **granulocytes**, which have prominent cytoplasmic granules, and **agranulocytes**, which lack them. There are three basic types of granulocytes: **neutrophils, basophils,** and **eosinophils.** The two types of agranulocytes are **monocytes** and **lymphocytes.** All leukocytes develop from bone marrow stem cells.

Neutrophils (Fig. 11.1A) are 12 μm–15 μm in diameter—about twice the size of an erythrocyte (RBC)[1] and are the most abundant WBCs in blood. Their most prominent cytoplasmic granules are neutral-staining and thus do not have the intense color of other granulocytes when prepared with Wright's or Giemsa stain. When needed, they leave the blood and enter tissues to phagocytize foreign material, whose recognition requires binding to a variety of different membrane receptors. Once internalized by phagocytosis, bacteria are killed by several mechanisms acting in concert, including oxidation of cell structures by oxygen radicals, enzymatic destruction of the cell wall, and leakage through the cell membrane caused by cationic peptides. Neutrophils typically live only a few days before undergoing apoptosis. Host cell debris, dead neutrophils, and dead bacteria form pus.

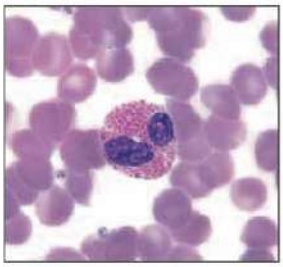
Mature neutrophils sometimes are referred to as **segs** because their nucleus usually is segmented into two to five lobes. Because of the variation in nuclear appearance, they also are called **polymorphonuclear neutrophils** (PMNs). Immature neutrophils lack this nuclear segmentation and are referred to as **bands** (Fig. 11.1B). This distinction is useful because a patient with an active infection increases neutrophil production, which leads to a higher percentage of immature band cells in the blood. The inactive X-chromosome is frequently seen as a "drumstick" extending from the neutrophil nucleus of females.

Eosinophils (Fig. 11.2) are 12 μm–15 μm in diameter (about twice the size of an RBC), generally have two lobes in their nucleus, and have red staining cytoplasmic granules. Most reside in connective tissues and are sometimes difficult to find in blood smears, where they represent less than 3% of all leukocytes. Their functions are varied and their numbers increase during allergic reactions, chronic inflammation, and parasitic infections. They phagocytose and digest antigen-anitbody complexes, and their secretions include chemicals that control the inflammatory response (e.g., histaminase), and cytotoxic and neurotoxic chemicals that attack parasites.

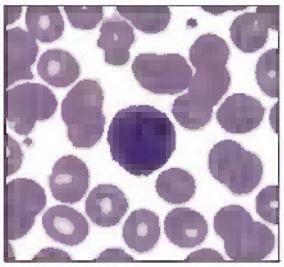
Basophils (Fig. 11.3) are the least abundant WBCs in normal blood and are difficult to find in normal blood. They are structurally and functionally similar to tissue



11.1 Neutrophils (Wright's Stain, ×1000) ◆ (A) The segmented nucleus of this cell identifies it as a mature neutrophil (seg). About 30% of neutrophils in blood samples from females demonstrate a "drumstick" protruding from the nucleus, as in this specimen (arrow). This is the region of the inactive X chromosome. (B) At the right is an immature band neutrophil (or slightly beyond the band stage, but still immature) with an unsegmented nucleus. Also shown is a monocyte (above left) and a lymphocyte (left).



11.2 Eosinophil (Wright's Stain, ×1000) ◆ These granulocytes are relatively rare and are about twice the size of red blood cells. Their cytoplasmic granules stain red, and their nucleus usually has two lobes.



11.3 Basophil (Wright's Stain, ×1000) ◆ Basophils comprise less than 0.2% of all white blood cells and require patience to find, because they are rare. (At 0.2%, you would theoretically have to look at 500 WBC just to see one.) They are slightly larger than red blood cells and have dark-purple cytoplasmic granules that obscure the nucleus. They release histamine and other chemicals that enhance the inflammatory response.

[1] It is convenient to discuss leukocyte size in terms of erythrocyte size because RBCs are so uniform in diameter. In an isotonic solution, erythrocytes are 7.5 μm in diameter.

mast cells and produce some of the same chemicals (histamine and heparin) as well as bind immunoglobin E (IgE), but are derived from different stem cells in bone marrow. They are 12 µm–15 µm in diameter and their dark-staining cytoplasmic granules usually obscure the one- or two-lobed nucleus.

Agranulocytes include monocytes and lymphocytes. Monocytes (Fig. 11.4) are the blood form of **macrophages**, which phagocytize foreign cells, cell debris, and also act as antigen-presenting cells during many immune responses. They are the largest of the leukocytes, being two to three times the size of RBCs (12 µm–20 µm). Their nucleus is horseshoe-shaped (or at least indented), and the cytoplasm lacks prominent granules, but may appear finely granular.

Lymphocytes (Fig. 11.5A) are cells of specific acquired immunity. They are approximately the same size as RBCs, or up to twice their size, and have very little cytoplasm visible around their spherical nucleus. Two functional types of lymphocytes are the **T-cell**, involved in cell-mediated immunity, and the **B-cell**, which converts to a **plasma cell** when activated, and produces antibodies. **Natural killer (NK) cells** comprise a third class of lymphocyte that kills foreign or virally-infected cells without antigen-antibody interaction (Fig. 11.5B). They are larger than B- and T-lymphocytes, being up to 16 µm and have large cytoplasmic granules, which accounts for their other name: **large granular lymphocytes.**

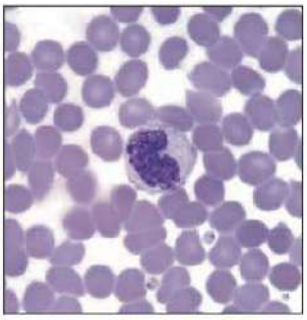
In a differential white cell count, a sample of blood is observed under the microscope, and at least 100 WBCs are counted and tallied (this task is automated now). Approximate normal percentages for each leukocyte are as follows and as summarized in Table 11-1.

neutrophils (mostly segs) 55%–65%
lymphocytes 25%–33%
monocytes 3%–7%
eosinophils 1%–3%
basophils <0.2%.

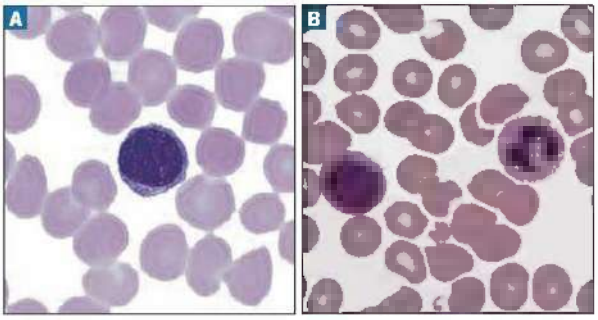
TABLE 11-1 Typical Features of Human Leukocytes in Blood

Table of Results

Cell	Abundance in Blood (%)	Diameter (µm)	Nucleus	Cytoplasmic Granules (Wright's or Giemsa Stain)	Functions
Granulocyte					
Neutrophil	55–65	12–15	2–5 lobes	Present, but stain poorly; contain antimicrobial chemicals	Phagocytosis and digestion of (usually) bacteria
Eosinophil	1–3	12–15	2 lobes	Present and stain red; contain antiparasitic chemicals and inflammatory moderators (e.g., histaminase)	Present in inflammatory reactions (removal of antigen-antibody complexes) and destruction of multicellular parasites (worms) with cytotoxins and neurotoxins
Basophil	<0.2	12–15	Unlobed or 2 lobes	Present and stain dark purple; contain histamine and other chemicals	Sustain inflammatory response by releasing chemicals, such as heparin (an anticoagulant) and histamine (as vasodilator)
Agranulocyte					
Lymphocyte	25–33	7–18 (rare)	Spherical (leaving little visible cytoplasm)	Absent	Active in specific acquired immunity (as T- and B-cells). Also active as NK cells.
Monocyte	3–7	12–20	Horseshoe-shaped (cytoplasm is prominent)	Absent	Phagocytosis and presentation of antigens to immune cells (as macrophages)



11.4 Monocyte (Wright's Stain, ×1000) ◆ Monocytes are the blood form of macrophages. They are about twice the size of red blood cells and have a round or indented nucleus.



11.5 Lymphocytes (Wright's Stain, ×1000) ◆ Lymphocytes are common in the blood, comprising up to 33% of all WBCs. **(A)** Small- and medium-sized lymphocytes are about the size of red blood cells and have only a thin halo of cytoplasm encircling their round nucleus. They belong to functional groups called B-cells and T-cells (which are morphologically indistinguishable). **(B)** About 15% of blood lymphocytes are large granular lymphocytes, most of which are natural killer (NK) cells (left). Also visible in this micrograph is a neutrophil.

Application

A differential blood cell count is done to determine approximate numbers of the various leukocytes in blood. Excess or deficiency of all or a specific group is indicative of certain disease states. Even though differential counts are automated now, it is good training to perform one "the old-fashioned way" using a blood smear and a microscope to get an idea of the principle behind the technique.

In This Exercise

You will be examining prepared blood smears and doing a differential count of white blood cells. As an optional activity, you may look at smears of abnormal blood and compare the differential count to normal blood.

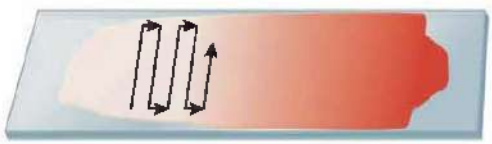
✓ Materials

Per Student Group

- ☐ Commercially prepared human blood smear slides (Wright's or Giemsa stain)
- ☐ (Optional) commercially prepared abnormal human blood smear slides (e.g., infectious mononucleosis, eosinophilia, or neutrophilia)

Procedure

1. Obtain a blood smear slide and locate a field where the cells are spaced far enough apart to allow easy counting. (The cells should be fairly dense on the slide, but not overlapping.)
2. Using the oil-immersion lens, scan the slide using the pattern shown in Figure 11.6. Be careful not to overlap fields when scanning the specimen. Choose a "landmark" blood cell at the right side of the field, and move the slide horizontally until that cell disappears off the left side. Avoid diagonal movement of the slide. As you scan, use the mechanical stage knobs separately to move the slide up and back or to the right and left in straight lines.
3. Make a tally mark in the appropriate box in the table on the data sheet, page 749, for the first 100 leukocytes you see.
4. Calculate percentages and compare your results with the accepted normal values.
5. Repeat with a pathological blood smear (if available).



11.6 Following a Systematic Path ◆ A systematic scanning path is used to avoid wandering around the slide and perhaps counting some cells more than once. Remember that a microscope image is inverted. If you want the image to move left, you must move the slide to the right.

References

Brown, Barbara A. *Hematology—Principles and Procedures*, 6th ed. Philadelphia: Lea and Febiger, 1993.

Diggs, L. W., Dorothy Sturm, and Ann Bell. *The Morphology of Human Blood Cells*, 4th ed. North Chicago, IL: Abbott Laboratories, 1978.

Mak, Tak W., Mary E. Saunders, and Bradley D. Jett. Chap. 2 in *Primer to the Immune Response*, 2nd ed. Burlington, MA: AP Cell, Elsevier, 2014.

Male, David, Jonathan Brostoff, David B. Roth, and Ivan M. Roitt. Chap. 2 in *Immunology*, 8th ed. Waltham, MA: Elsevier, 2013.

Mescher, Anthony L. Chap. 12 in *Junqueira's Basic Histology, Text and Atlas*, 12th ed. New York: McGraw Hill, Lange, 2010.

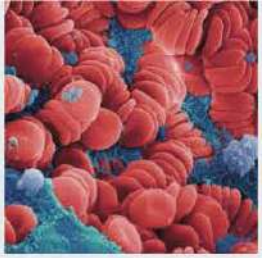
Ross, Michael H. and Wojciech Pawlina. Chap.10 in *Histology A Text and Atlas*, 6th ed. Philadelphia: Wolters Kluwer, Lippincott Williams & Wilkins., 2011.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 11-1

Differential Blood Cell Count

OBSERVATIONS AND INTERPRETATIONS

1 Record your data from the differential blood cell count in the table below. As you count the 100 white blood cells, make tally marks in the appropriate boxes. Then calculate the percentages of each type and compare them to the expected values.

Normal Blood						
	Monocytes	Lymphocytes	Segmented Neutrophils	Band Neutrophils	Eosinophils	Basophils
Number						
Percentage						
Expected percentage	3–7%	25–33%	55–65%	—	1–3%	<0.2%

2 If available, count 100 white blood cells as in step 1 above with an abnormal blood smear.

Abnormal Blood (Condition: _____)						
	Monocytes	Lymphocytes	Segmented Neutrophils	Band Neutrophils	Eosinophils	Basophils
Number						
Percentage						
Expected percentage	3–7%	25–33%	55–65%	—	1–3%	<0.2%

QUESTIONS

1 *How do the percentages of each WBC compare to the published values? What might account for any differences you noted?*

2 *If you did differential counts on abnormal blood, how did the percentages compare to normal blood? How can any differences you noted be explained in the context of the disease and/or defense process? Be sure to name the blood condition in your answer.*

3 *What is the purpose of scanning the slide in a systematic pattern (as described in the text)?*

4 *Most leukocytes function outside of the blood stream. Given this fact, why are they found in blood smears?*

Introduction to Antigens and Antibodies

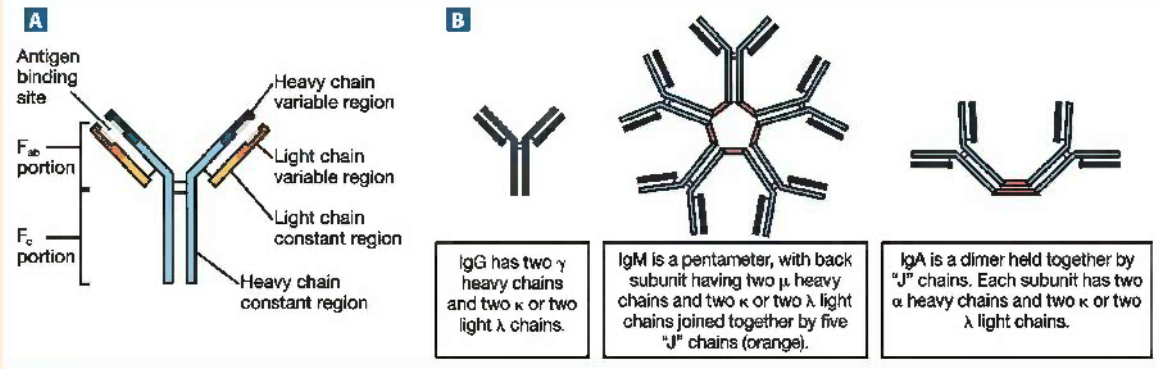
The body's defenses against infectious agents can be divided into nonspecific defenses (also called innate immunity) and specific acquired immunity. The former include external structures, such as epithelial barriers; chemical secretions, such as stomach acid and mucus; and mechanical properties, such as peristalsis and ciliary action. Nonspecific internal defenses include phagocytosis, the complement system, fever, inflammation, and interferon. All of these are categorized as nonspecific because their activation and performance is generic; for instance, phagocytic cells are not individually dedicated to a particular infectious agent, nor do interferon molecules each target a different virus. On the other hand, the specific acquired immune system involves the production of **antibodies** or **immune cells** that react with a single **antigen**. Because the following lab exercises utilize antibodies and not immune cells our focus will be on antibodies.

Using a simple definition, antigens are high molecular weight molecules with a definite three-dimensional shape that the body recognizes as being "foreign." More importantly, antigens stimulate the production of specific antibodies (or immune cells) and will react specifically with them once produced. In general, proteins make the best antigens, followed by polysaccharides. Lipids and nucleic acids do not make good antigens.

Antibodies are glycoproteins (**immunoglobins–Ig**) and each is composed of two identical long polypeptides called **heavy chains** and two identical shorter polypeptides called **light chains** (Fig. 11.7A). Functionally, each antibody molecule behaves as if it has the shape of the letter "Y." There are two antigen binding sites, one at the end of each arm of the "Y," formed by one heavy and one light chain in combination. This part of the antibody is called the **F_{ab} portion**. The stem of the "Y" (the **F_c** portion) is formed by the remainder of the two heavy chains and performs **effector functions**, such as binding to phagocytic cells or activating the complement system.

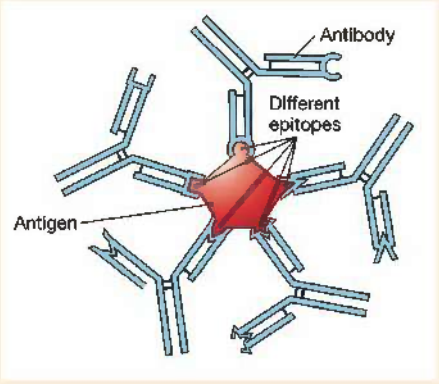
Obviously, there must be great antibody diversity in order to protect against all the antigens the world presents. On a gross level, antibody diversity begins with the type of heavy chain present. The five basic immunoglobin classes (with heavy chains in parentheses) are: IgG (γ), IgM (μ), IgA (α), IgD (δ), and IgE (ε). Additional diversity occurs because light chains come in two forms: kappa (κ) and lambda (λ), which are able to combine with any of the heavy chains. Further, antibodies may be joined into more complex arrangements, such as in IgM, where five Y-shaped antibodies form a pentamer, and in IgA that exists as a dimer in mucus and other secretions (Fig. 11.7B).

Much of the remainder of antibody diversity occurs as a result of each lymphocyte's ability to produce its own version of its heavy and light chains. Each heavy and light chain has an amino acid sequence along most of its length that is more or less characteristic of its type. This is called the **constant region**. However, each heavy and light chain also contains a **variable region**, which differs in amino acid sequence from those produced by all other lymphocytes and from each other. When assembled, the variable regions combine to form a unique three-dimensional pocket that will bind an antigen with a complementary shape.


A
Antigen binding site
F_{ab} portion
F_c portion
Heavy chain variable region
Light chain variable region
Light chain constant region
Heavy chain constant region
B
IgG has two γ heavy chains and two κ or two light λ chains.
IgM is a pentameter, with back subunit having two μ heavy chains and two κ or two λ light chains joined together by five "J" chains (orange).
IgA is a dimer held together by "J" chains. Each subunit has two α heavy chains and two κ or two λ light chains.

11.7 Antibody Structure ♦ (**A**) The fundamental antibody structure is two identical heavy chains and two identical light chains held together by covalent disulfide bonds. Each chain has a constant region and a variable region composed of "standard" and unique amino acid sequences. The variable regions are unique to each B lymphocyte and join together to form the antigen binding site, thus accounting for the ability of each B-cell to produce antibodies that react with only a single antigen (epitope). (**B**) There are five antibody classes identified by the heavy chain in each. Some antibodies combine to form more complex arrangements than the simple "Y" shape. IgM and IgA are two examples.

Molecules have a three-dimensional shape and a surface topography. Because antigens have a relatively unchanging three-dimensional shape with a varied surface topography, different parts of an antigen can stimulate production of different antibodies, each reacting with a different surface feature. These surface features are called **epitopes**, and their presence makes an immune response to any antigen molecule more complex than just one antibody for one antigen (Fig. 11.8).



11.8 Epitopes ♦ Shown is an artist's representation of an antigen with multiple epitopes. Each epitope has the ability to stimulate production of antibodies that will bind them because of the complementary shapes. Often biologists refer to the whole molecule (and sometimes even the whole cell or virus particle) as an "antigen," but in reality the immune system "sees" only epitopes. In this illustration, a single molecule has resulted in the production of multiple antibodies targeting it.

Simple Serological Reactions

Antigen–antibody reactions are highly specific and occur in vitro as well as in vivo. Serology is the discipline that exploits this specificity as an in vitro diagnostic tool. All serological tests can be designed to identify antigen or antibody in a sample. The choice for any particular test largely depends on the circumstances of the disease process; that is, whether it is easier to identify antigens or antibodies in a patient sample. All serological tests also fundamentally rely on the reaction between antigen and homologous antibody, but they differ in the way a positive reaction is indicated. For instance, two simple serological reactions—agglutination and precipitation—are used in the following four exercises because they result in the formation of complexes that can be viewed with the naked eye and without sophisticated equipment.

The precipitin ring test (Exercise 11-2) and double gel immunodiffusion (Exercise 11-3) illustrate precipitation. Both may be used to identify antigens or antibodies in a sample; the latter also is used to compare antigens in more than one sample. The slide agglutination test (Exercise 11-4) can be an important diagnostic (and highly specific) tool for the identification of organisms. It is especially useful for serotyping large genera such as *Salmonella*. Hemagglutination, a type of agglutination reaction, detects specific antigens on red blood cells, and is the standard test for determining blood type (Exercise 11-5). The most sophisticated serological test covered in this section is in Exercise 11-6. It is an ELISA designed to detect antibody in a sample. Its indicator reaction is the production of a colored product from a colorless substrate by an enzyme attached to a secondary antibody that is present in the reaction mixture only if it has bound to the antibody being looked for in the patient sample. ♦

EXERCISE
11-2

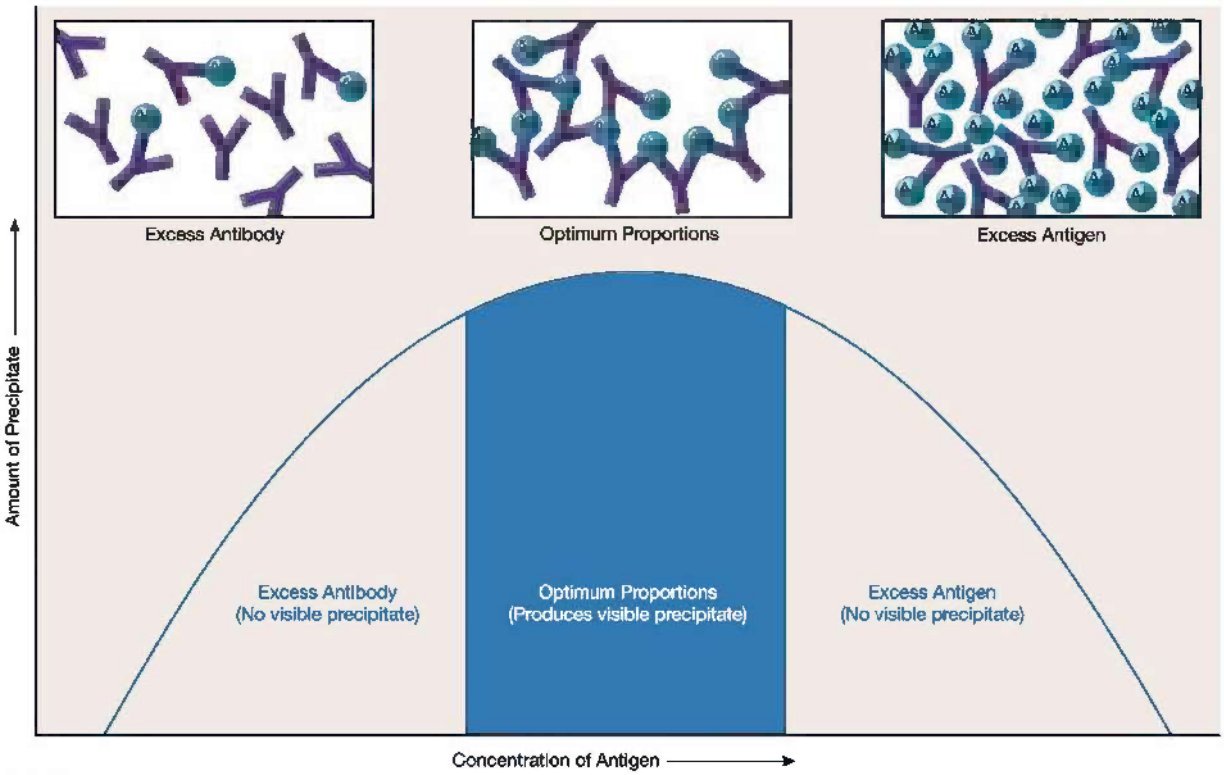
Precipitin Ring Test

Theory

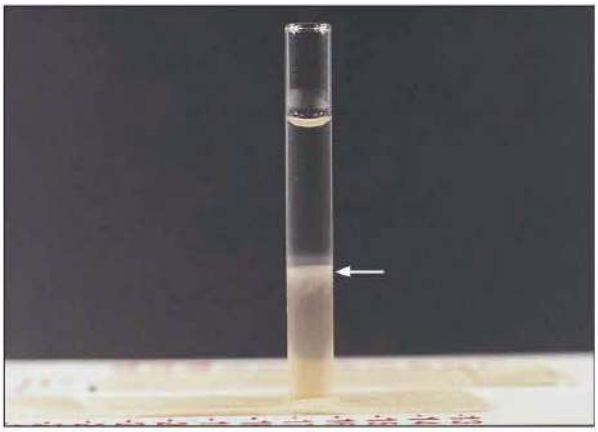
Soluble antigens may combine with **homologous antibodies** to produce a visible **precipitate**. Precipitate formation in vivo serves to inactivate toxins or enzymes produced by infectious agents. The precipitates can subsequently be cleared by cells such as eosinophils. Precipitate formation in vitro can serve as evidence of antigen–antibody reaction in a diagnostic test and is considered to be a positive result.

Precipitation is produced because each antibody has (at least) two antigen binding sites and many antigens have multiple epitopes. This results in the formation of a complex lattice of antibodies and antigens and produces the visible precipitate—a positive result. As shown in Figure 11.9, if either antibody or antigen is found in a concentration that is too high relative to the other, no visible precipitate will be formed, even though both are present, and produces a false negative result. (Of course, the microbiologist would record a negative result and would be unaware that it is truly positive and that the test has produced a false negative result.) **Optimum proportions** of antibody and antigen are necessary to form precipitate, and they occur in the **zone of equivalence.**

Several styles of precipitation tests are used. The precipitin ring test is performed in a small test tube or a capillary tube. Antiserum (containing antibodies homologous to the antigen being looked for) is placed in the bottom of the tube. The sample with the suspected antigen is layered on the surface of the antiserum in such a way that the two solutions have a sharp interface. As the two fluids diffuse into each other, precipitation occurs where optimum proportions of antibody and antigen are found (Fig. 11.10). This test also may be run to test for antibody in a sample.



11.9 Precipitation Reactions ◆ Precipitation occurs between soluble antigens and homologous antibodies where they are found in optimal proportions to produce a cross-linked lattice. Excess antigen or excess antibody prevents substantial cross-linking, so no lattice is formed and no visible precipitate is seen—even though both antigen and antibody are present. The test would then be recorded as negative, even though it is really positive. In this graph, antibody concentration is kept constant as antigen concentration is adjusted.



11.10 Positive Precipitin Ring Test ◆ A sample of antigen has been layered over an antiserum. The white precipitation ring has formed at the site of optimum proportions of antibodies and antigens (arrow).

Application

Precipitation reactions can be used to detect the presence of either antigen or antibody in a sample. They have mostly been replaced by more sensitive serological techniques for diagnosis but are still useful as a simple demonstration of serological reactions.

In This Exercise

You will perform a simple serological precipitation test to illustrate homology between equine albumin and equine albumin antiserum.

✓ Materials

Per Student Group

- ☐ Small test tube rack
- ☐ Two clean 6 × 50 mm Durham tubes
- ☐ Equine serum (containing equine albumin)
- ☐ Equine albumin antiserum (containing equine albumin antibodies)
- ☐ 0.9% saline solution
- ☐ Three small transfer pipettes with a fine tip
- ☐ (Optional) bovine serum and bovine albumin antiserum may be substituted

11

Procedure

1. With a transfer pipette, carefully add equine antiserum to both Durham tubes. Fill from the bottom of the tube until it is about 1/3 full. Place both tubes upright in test tube rack.
2. Mark one tube A. Use a fresh transfer pipette to add the equine serum in such a way that a sharp and distinct second layer is formed without any mixing of the two solutions. It is critical not to allow any mixing. Success usually can be achieved by allowing the serum to trickle slowly down the inside of the glass (Fig. 11.11).
3. Mark the second tube B. Add the 0.9% saline in the same way you added equine serum to tube A. Use a fresh transfer pipette.
4. Incubate both at 35 ± 2°C undisturbed for 1 hour. (Often, a positive result is seen sooner.)
5. Observe the tubes for the characteristic ring formed at the interface of the two solutions (zone of optimum proportions). If after 1 hour there is no ring in either tube, place both in the refrigerator for 12 to 24 hours and then recheck.
6. Draw and label the tubes after incubation in the space provided on the data sheet.

11.11 Layering the Antigen on Top of the Antibody ◆ Add antigen to the tube by placing the pipette into the tube about 5 mm from the antiserum. Let the antigen trickle down the inside of the glass. It is important that there is no mixing of the two solutions. (Note the interface indicated by the arrow.) Allow the tube to stand undisturbed for up to one hour.

References

Lam, Joseph S. and Lucy M. Mutharia. Page 120 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

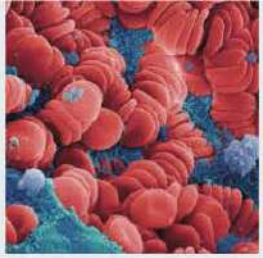
Mak, Tak W., Mary E. Saunders, and Bradley D. Jett. Pages 621–622 in *Primer to the Immune Response*, 2nd ed. Burlington, MA: AP Cell, Elsevier, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 11-2

Precipitin Ring Test

OBSERVATIONS AND INTERPRETATIONS

1 Draw the tubes after incubation. Label the solutions in each and any precipitation lines.

QUESTIONS

1 *What was the purpose of incubating 0.9% saline over equine albumin antiserum in tube B? Be specific.*

2 *In general terms, describe how equine albumin antiserum could be obtained. What animal is least likely to be a source for it?*

3 *Suppose you were instructed to make two antiserum solutions: The first is identical to what you used in the lab exercise. The other is a 10^{-6} dilution of the antiserum. After incubation with the antigen, the full-strength antiserum produces a precipitin ring, but the diluted antiserum does not. Explain these results. Is this due to poor specificity or sensitivity of the test?*

4 *Suppose you were instructed to repeat this experiment, again using equine albumin antiserum in two tubes. You layer equine serum (containing equine albumin) over the antiserum in one tube and pig serum (containing pig albumin) over the antiserum in the other tube. After incubation, you see precipitin rings in both tubes. Explain these results. Is this due to poor specificity or sensitivity of the test?*

Double Gel Immunodiffusion

EXERCISE 11-3

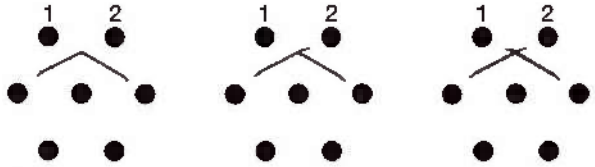
Theory

Precipitation reactions occur between soluble antigens and homologous antibodies. The precipitate forms when the two are at concentrations that allow maximum cross-linking in the antigen–antibody complex that forms (Fig. 11.9). The formation of a precipitate in vivo can neutralize toxins or enzymes produced by infectious agents. In this exercise, we use in vitro precipitate formation as an indicator of antigen and antibody reaction.

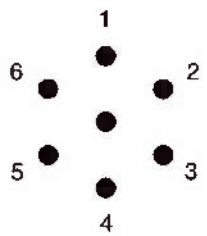
In its simplest form, **gel immunodiffusion** involves two wells formed in a saline agar plate. In one well is the antiserum; in the other is the sample with unknown antigen composition. The two diffuse out of their respective wells toward each other. If the sample has an antigen that will react with the antibody, then a precipitation line will form at the region of optimal proportions as their diffusion paths pass. If no precipitation line forms, the test is considered negative. That is, the sample has no antigen that will react with the antibodies. This procedure may also be used to identify the presence of antibody in a sample if a known antigen is used.

The diffusion path out of the well is not linear. Rather, it is radial in all directions. In a more complex form of immunodiffusion test, several wells are placed around a center well (Fig. 11.12). Antiserum (which usually contains antibodies that bind specifically with the different epitopes on the antigen molecule) is placed in the center well. Samples of known or unknown antigen (epitope) composition are put in the surrounding wells. Because diffusion out of the center well is radial, precipitation lines may form between the center well and any or all of the surrounding wells, depending on their epitope composition. The precipitation line pattern between neighboring wells is indicative of antigen relatedness in those wells (Fig. 11.13). A single, smooth, curved precipitation line indicates the two antigens in neighboring wells have identical epitopes (**identity**). Two spurs indicate unrelated antigens that share no epitopes (that the test can detect) and is considered **nonidentity** because two completely separate precipitation lines formed. A single spur indicates that the antigens share some, but not all, epitopes (**partial identity**) because two lines formed—a line of identity and a line of nonidentity.

In this exercise, we will simulate degrees of relatedness by making mixtures of very different antigens with mixtures of antiserum.



11.13 Precipitation Patterns and Antigen Relatedness (Compare with Figure 11.14) ◆ This diagram shows three possible precipitation patterns formed between the antibody in the center well and antigens in wells 1 and 2. The pattern on the left demonstrates identity between the antigens; that is, they have identical epitopes. The pattern in the middle demonstrates partial identity, which means that the antigens are related but not identical (they share some, but not all, epitopes). The pattern on the right shows nonidentity; the antigens do not share any epitopes (at least to the extent the test's sensitivity allows us to determine) and are not related.



11.12 Double Gel Immunodiffusion Well Template ◆ Use this template as a guide when boring the wells in the saline agar plate.

Application

Gel immunodiffusion tests can be used to identify the presence of antigen or antibody in a sample. They also are used to check samples for identical, related, or unrelated antigens based on their epitopes (Fig. 11.14). They also may be used to identify systemic fungal pathogens grown in culture.

In This Exercise

You will be performing gel radial immunodiffusion, a technique used to compare antigen relatedness, that is, epitope composition. Precipitation line patterns that illustrate antigen identity, nonidentity, and partial identity will be seen. In a real life situation, two antigens would be compared to see if they share one or more epitopes, but we will produce the various patterns artificially by mixing different combinations of horse and cow albumin and having them combine with a mixture of antisera to both horse and cow albumin.

11.14 Diagram Showing Antigen Relatedness (Compare with Figure 11.13) ◆ Shown are three pairs of antigens, each with four epitopes, that could be placed in adjacent wells of a double gel immunodiffusion test. In each pair, the antigen on the left is the same. **(A)** In this pair, the two antigens have identical epitopes and would produce a line of identity. **(B)** This pair of antigens shares two epitopes, but differ in the other two. They would show a pattern of partial identity. **(C)** This pair of antigens has no epitopes in common and would produce a pattern of nonidentity.

✓ Materials

Per Student Group

- ☐ One saline agar plate
- ☐ 3 mm punch (a glass dropper with a 3 mm diameter tip will work)
- ☐ Template for cutting wells (Fig. 11.12)
- ☐ Bovine antiserum (containing bovine albumin antibodies)
- ☐ Equine antiserum (containing equine albumin antibodies)
- ☐ 10% bovine serum (prepared by adding 9 drops physiological saline to 1 drop 100% serum)
- ☐ 10% equine serum (prepared by adding 9 drops physiological saline to 1 drop 100% serum)
- ☐ Physiological saline solution (0.9% saline)
- ☐ Disposable micropipettes
- ☐ Two small test tubes

Procedure

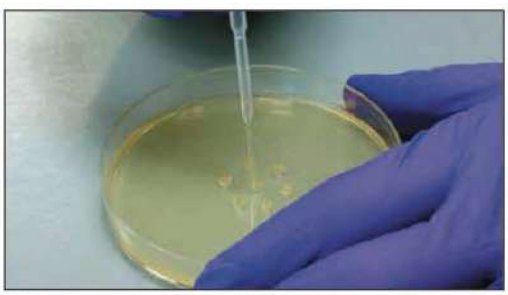
Lab One

1. Center the saline agar plate over the template in Figure 11.12.
2. Using the punch or glass dropper, cut the seven wells in the agar as shown in Figure 11.15. If the small agar disks do not come out with the dropper, use suction with the dropper and bulb to dislodge and remove them. Avoid lifting the agar when removing the punched-out disks.
3. Number the peripheral wells 1 through 6 as shown in Figure 11.12.
4. In a small test tube or mixing cup prepare a 1:1 mixture of equine albumin antiserum and bovine albumin antiserum. In a second test tube, make a 1:1 mixture of equine and bovine serum.
5. Using a *different* micropipette for each transfer and the technique illustrated in Figure 11.16, carefully fill the wells as indicated below. Be careful when adding the solutions not to overfill the wells. One or two drops are usually enough.
   a. Fill the center well with the equine/bovine antiserum mixture.
   b. Fill wells 1 and 2 with 10% equine serum.
   c. Fill wells 3 and 4 with 10% bovine serum.
   d. Fill well 5 with 0.9% saline.
   e. Fill well 6 with the equine/bovine serum mixture.
6. Cover the plate and allow it to sit undisturbed for 30 minutes.
7. Incubate at room temperature for up to 72 hours or until precipitation lines appear.

Lab Two

1. Examine the plate for precipitation lines.
2. Record your results on the data sheet, page 759. For each pair of wells, interpret the precipitation patterns.

11.15 Boring Wells in the Saline Agar Plate ◆ When cutting wells in the agar, press straight down; *do not twist*. Twisting the cutter or dropper may create fissures in the agar, which could disrupt the diffusion of the solutions.



11.16 Filling the Wells ◆ Place the tip of the pipette in the bottom of the well. Fill slowly to prevent creating air bubbles and to minimize spilling serum over the sides.

References

Lam, Joseph S. and Lucy M. Mutharia. Page 120 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Mak, Tak W., Mary E. Saunders, and Bradley D. Jett. Pages 621–623 in *Primer to the Immune Response*, 2nd ed. Burlington, MA: AP Cell, Elsevier, 2014.

Ouchterlony, O. Page 20 in *Handbook of Immunodiffusion and Immunoelectrophoresis*. Ann Arbor, MI: Ann Arbor Science Publishers, 1968.

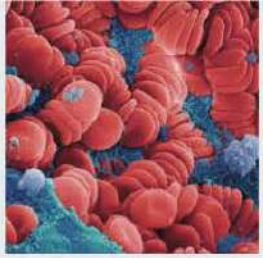
Tille, Patricia M. Pages 134–135 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

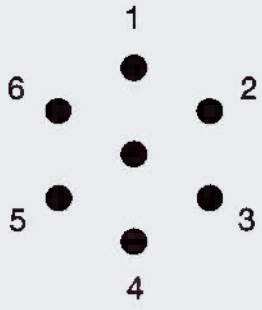


DATA SHEET 11-3

Double Gel Immunodiffusion

OBSERVATIONS AND INTERPRETATIONS

1 Draw and interpret the results of your immunodiffusion plate in the diagram below. (*Note:* Do not use this as the template for the wells in your plate; use Figure 11.12.)



QUESTIONS

1 *What was the purpose of well 5? Be specific.*

2 *How many lines of identity did your plate demonstrate?*

3 *Between which two wells did you see nonidentity?*

4 *Between which two wells did you see partial identity?*

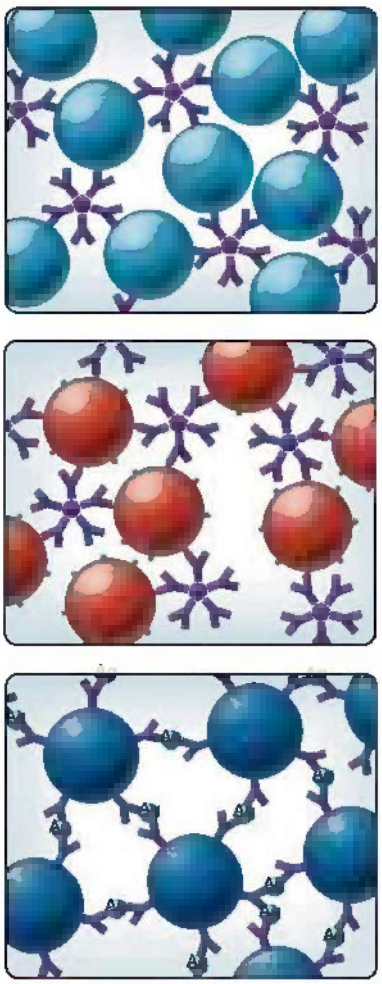
5 *Examine Figure 11.9. Suppose you performed double gel immunodiffusion and all you got was the Zone of Antigen Excess. Is this a false positive or a false negative result? Is it due to poor specificity or sensitivity of the test?*

6 *Suppose you performed the double gel immunodiffusion test again using the procedure in this exercise. How could you explain the presence of two precipitation lines between well 1 and the center well?*

EXERCISE
11-4
Slide Agglutination

Theory

Particulate antigens (such as whole cells) may combine with homologous antibodies to form visible clumps called **agglutinates**. In vivo, these agglutinates immobilize the cells until they can be removed by phagocytic cells. In vitro, **agglutination** can serve as evidence of antigen–antibody reaction and is considered a positive result. Agglutination reactions are highly sensitive and may be used to detect either the presence of antigen or antibody in a sample.



Direct agglutination occurs with naturally particulate antigens. Either antigen or antibody may be detected in a sample using this style of agglutination test.

Detection of antibody in a sample can be done by indirect agglutination. A test solution is prepared by artificially attaching homologous antigen (blue) to a particle (red) such as red blood cells or latex beads and mixing with the sample suspected of containing the antibody (purple).

Another style of indirect agglutination detects antigen (light blue) in a sample. Antibodies (purple) are artificially attached to particles (dark blue), which are then mixed with the sample suspected of containing the antigen.

11.17 Direct and Indirect Agglutination Tests ◆ Direct agglutinations involve naturally particulate antigens. Indirect agglutination relies on attaching either the antigen or antibody to a particle, such as a latex bead or red blood cell.

There are many variations of agglutination tests (Fig. 11.17). **Direct agglutination** relies on the combination of antibodies and naturally particulate antigens. **Indirect agglutination** relies on artificially constructed systems in which agglutination will occur. These involve coating particles (such as RBCs or latex microspheres) with either antibody or antigen, depending on what is being looked for in the sample. Addition of the homologous antigen or antibody then will result in clumping of the artificially constructed particles and turns what would have naturally been a less sensitive precipitation reaction into a more sensitive agglutination. Agglutination tests may be performed in test tubes, microtitre plates, or on microscope slides. Some agglutinations also allow determination of antibody titre in the patient sample.

Application

Agglutination reactions may be used to detect the presence of either antigen or antibody in a sample. Direct agglutination reactions are used to diagnose some diseases, determine if a patient has been exposed to a certain pathogen, and are involved in blood typing. Indirect agglutination is used in some pregnancy tests as well as in diagnosing disease.

In This Exercise

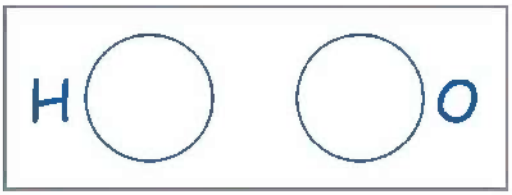
You will perform a simple agglutination test to identify the presence of *Salmonella* H antigen in a sample. You will compare this reaction to a reaction between the nonhomologous *Salmonella* anti-H antiserum and the *Salmonella* O antigen.

✓ Materials

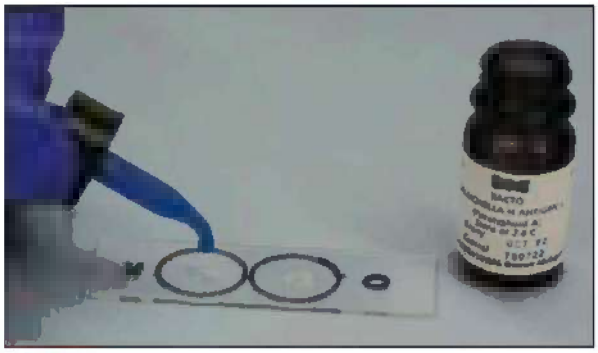
Per Student Group

- ☐ One clean microscope slide
- ☐ Two toothpicks
- ☐ Marking pen
- ☐ *Salmonella* H antigen
- ☐ *Salmonella* O antigen
- ☐ *Salmonella* anti-H antiserum

11



11.18 Prepare the Slide ◆ With your marking pen, draw two dime-size circles on the slide. Label one circle "H" and the other "O." The circles will be where you check for the presence of *Salmonella* H and O antigens, respectively.



11.19 Adding the Antigen ◆ Carefully add the H and O antigens to the drops of anti-H antiserum already on the slide. Do not touch the dropper to the antiserum.

Procedure

1. Using a marking pen, draw two circles approximately the size of a dime on a microscope slide (Fig. 11.18). Label one "O" and the other "H."
2. Place a drop of *Salmonella* anti-H antiserum in each circle.
3. Place a drop of *Salmonella* O antigen in the "O" circle and a drop of *Salmonella* H antigen in the "H" circle (Fig. 11.19). Be careful not to touch the dropper to the antiserum already on the slide.
4. Using a *different* toothpick for each circle, mix until each antigen is completely emulsified with the antiserum. Do not over-mix. Discard the toothpicks in a sharps container.
5. Gently rock this slide for a few minutes and observe for agglutination. Record your observations on the data sheet, page 763. (When rocking, the fluid should not spread. If it does, you are rocking too hard.)

Alternative Test Procedure

Your instructor will cover the labels of the two *Salmonella* antigen bottles. You will use this procedure to identify which one contains the *Salmonella* H antigen.

1. Using a marking pen, draw two circles approximately the size of a dime on a microscope slide (Fig. 11.18).
2. Place a drop of one *Salmonella* unknown antigen in one circle and a drop of the other *Salmonella* unknown antigen in the other circle (Fig. 11.19).
3. Place a drop of *Salmonella* anti-H antiserum in each circle. Be careful not to touch the dropper to the antigen solutions already on the slide.
4. Using a *different* toothpick for each circle, mix until each antigen is completely emulsified with the antiserum. Do not *over*-mix. Discard the toothpicks in a biohazard container.
5. Gently rock this slide for a few minutes, then observe for agglutination. Record your observations on the data sheet.

References

Carpenter, A. Betts. Pages 63–64 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Collins, C. H., Patricia M. Lyne, J. M. Grange. Page 118 in *Collins and Lyne's Microbiological Methods,* 7th ed. Oxford, Boston: Butterworth-Heinemann, 1995.

Khanna, Raj. Pages 363–366 in *Immunology.* New Delhi, India: Oxford University Press. 2011.

Lam, Joseph S. and Lucy M. Mutharia. Page 120 in *Methods for General and Molecular Bacteriology.* Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

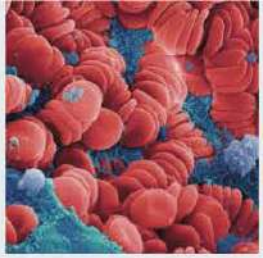
Tille, Patricia M. Pages 135–136 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis, MO: Mosby, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

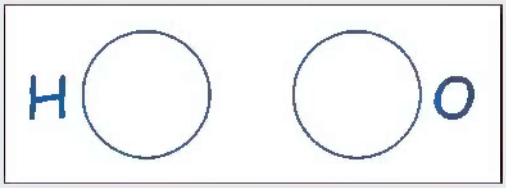


DATA SHEET 11-4

Slide Agglutination

OBSERVATIONS AND INTERPRETATIONS

1 Sketch and label your results in the diagram below.



QUESTIONS

1 *What is the purpose of performing this experiment with both H and O antigens?*

2 *Suppose you performed this test and got agglutination in both samples. Eliminating contamination of the antigen and antiserum samples as a possibility, provide an explanation of this hypothetical result. Is this attributable to poor sensitivity or specificity of the test system?*

3 *Suppose you performed this test and neither sample produced agglutination even though you know the antigens and antibodies* should *react. Would this be attributable to poor sensitivity or specificity of the test system?*

4 *Higher vertebrates produce antibodies;* Salmonella *is a prokaryote. Given these facts, how can* Salmonella *antiserum be produced?*

EXERCISE 11-5

Blood Typing

Theory

Hemagglutination is a general term applied to any agglutination test in which clumping of red blood cells indicates a positive reaction. Blood tests as well as a number of indirect diagnostic serological tests are hemagglutinations.

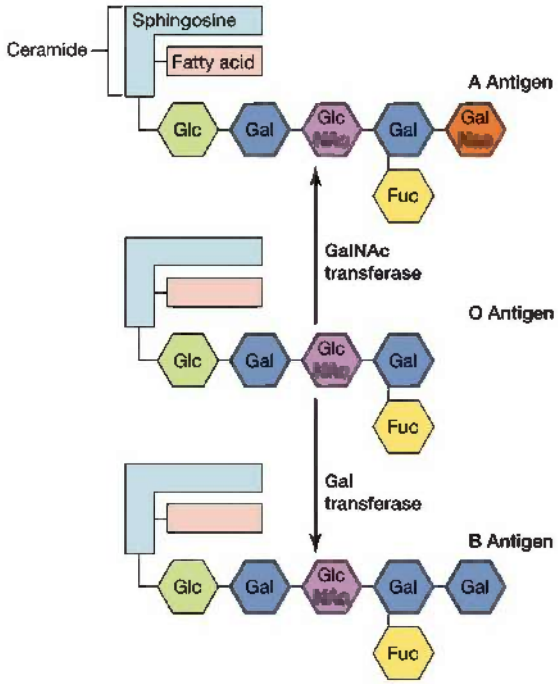
The most common form of blood typing detects the presence and absence of **A** and/or **B antigens** on the surface of red blood cells. An individual with type A blood has RBCs with the A antigen and produces anti-B antibodies. Conversely, an individual with type B blood has RBCs with the B antigen and produces anti-A antibodies. People with type AB blood have *both* A and B antigens on their RBCs and lack anti-A and anti-B antibodies. Type O individuals lack A and B antigens but produce *both* anti-A and anti-B antibodies.

While genetically determined, the A and B antigens are not direct gene products, but rather are produced by enzymatic reactions that modify the O antigen, a short (5-sugar) polysaccharide, found on all RBC membranes (Fig. 11.20). People with type A blood produce an enzyme that adds the sugar N-acetylgalactosamine to the end of the O antigen to make the A antigen. In like fashion, people with type B blood have a different enzyme that adds the sugar galactose to the end of the O antigen. People with type AB blood make both enzymes and modify the O antigen in both ways, and people with type O blood can only synthesize the O antigen, which remains unmodified.

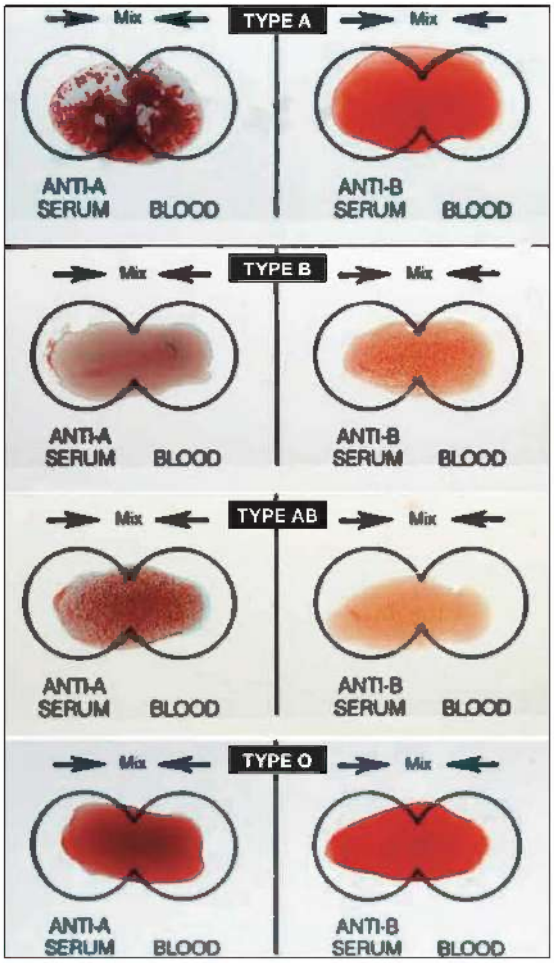
ABO blood type is ascertained by adding a patient's blood separately to anti-A and anti-B antiserum and observing any signs of agglutination (Table 11-2 and Fig. 11.21). Agglutination with anti-A antiserum indicates the presence of the A antigen and type A blood. Agglutination with anti-B antiserum indicates the presence of the B antigen and type B blood. If both agglutinate, the individual has type AB blood; lack of agglutination occurs in individuals with type O blood.

TABLE **11-2** **Interpretation of Blood Typing Results**

Reaction With					
Anti-A Antiserum	**Anti-B Antiserum**	**Anti-Rh Antiserum**	**Interpretation**	**Symbol**	**(Possible) Genotype**
Agglutination	No Agglutination	Agglutination	A antigen present B antigen absent Rh antigen present	A+	I^AI^A or I^Ai and *DD* or *Dd*
Agglutination	No Agglutination	No Agglutination	A antigen present B antigen absent Rh antigen absent	A−	I^AI^A or I^Ai and *dd*
No Agglutination	Agglutination	Agglutination	A antigen absent B antigen present Rh antigen present	B+	I^BI^B or I^Bi and *DD* or *Dd*
No Agglutination	Agglutination	No Agglutination	A antigen absent B antigen present Rh antigen absent	B−	I^BI^B or I^Bi and *dd*
Agglutination	Agglutination	Agglutination	A and B antigens present Rh antigen present	AB+	I^AI^B and *DD* or *Dd*
Agglutination	Agglutination	No Agglutination	A and B antigens present Rh antigen absent	AB−	I^AI^B and *dd*
No Agglutination	No Agglutination	Agglutination	A and B antigens absent Rh antigen present	O+	*ii* and *DD* or *Dd*
No Agglutination	No Agglutination	No Agglutination	A and B antigens absent Rh antigen absent	O−	*ii* and *dd*



11.20 The A and B Blood Antigens ♦ All humans produce the short oligosaccharide that produces type O blood. However, if a person has at least one gene (either genotype I^AI^A or I^Ai) to produce the enzyme *N*-acetylgalactosamine transferase (GalNAc transferase) they add the sugar *N*-acetylgalactosamine to the O oligosaccharide and have type A blood. If a person has at least one allele (either genotype I^BI^B or I^Bi) to produce the enzyme galactose transferase (Gal transferase) they add the sugar galactose to the O oligosaccharide and have type B blood. The presence of both enzymes (genotype I^AI^B) results in both reactions occurring and type AB blood. The absence of both enzymes (genotype *ii*) leaves the O oligosaccharide unmodified and the person has type O blood. Because it is present in all humans, it is not detected as an antigen.



11.21 ABO Blood Groups ♦ Blood typing relies on agglutination of RBCs by anti-A and/or anti-B antisera. The blood types are as shown.

A similar test is used to determine the presence or absence of the **Rh factor**, an erythrocyte membrane polypeptide. There are actually three Rh antigens (C, D, and E), and possession of any one of them makes the person Rh positive, but the one usually detected in blood testing is the D antigen. If clumping of the patient's blood occurs when mixed with anti-D antiserum, the patient is Rh positive.

The ABO and Rh blood factors are genetically unrelated, so any combination of the two is possible (e.g., "O–positive" or "O–negative"). The A and B blood factors are determined by the codominant I^A and I^B alleles, respectively. The recessive *i* allele, which all humans have, produces the molecule to which the A and/or B antigen(s) attach. The Rh factor is most simply viewed as being the product of a dominant allele *D*, with the recessive *d* allele producing no Rh factor. See Table 11-2 for the possible genotypes for each blood type.

Application

Blood typing is a simple example of an agglutination test using blood. Clinically, it is used for cross-matching donor and recipient blood prior to transfusion. It is also used to prophylactically treat Rh-negative mothers carrying Rh-positive fetuses to prevent erythroblastosis fetalis in that or any subsequent Rh-positive fetus.

In This Exercise

You will determine your blood type with respect to two different markers: the ABO group and the Rh factor. Then, you will compile class data and compare it to blood type frequencies in the United States (Table 11-3).

TABLE **11-3** Approximate Percentages of Blood Types in the United States

Blood Type	Caucasian	African American	Hispanic	Asian
O+	37	47	53	39
O−	8	4	4	1
A+	33	24	29	27
A−	7	2	2	0.5
B+	9	18	9	25
B−	2	1	1	0.4
AB+	3	4	2	7
AB−	1	0.3	0.1	0.1

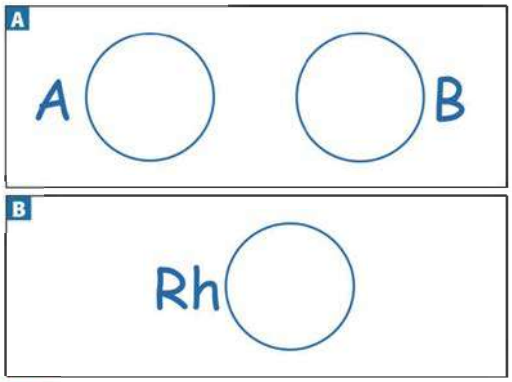
✓ Materials

Per Student Group

- ☐ Blood typing anti-A antiserum
- ☐ Blood typing anti-B antiserum
- ☐ Blood typing anti-Rh (anti-D) antiserum
- ☐ Two microscope slides
- ☐ Three toothpicks
- ☐ Marking pen
- ☐ Sterile lancets
- ☐ Alcohol wipes
- ☐ Small adhesive bandages
- ☐ Sharps container
- ☐ Disposable gloves

Procedure

1. With your marking pen, draw two circles on one microscope slide. Label one circle "A" and the other "B" (Fig. 11.22A).
2. Draw a single circle with your marking pen in the center of a second microscope slide. Label it "Rh" (Fig. 11.22B).
3. Place a drop of anti-A antiserum in the "A" circle.
4. Place a drop of anti-B antiserum in the "B" circle.
5. On the second microscope slide, place a drop of anti-D antiserum.
6. Clean the tip of your finger with an alcohol wipe. Let the alcohol dry.
7. Open a lancet package and remove the lancet, being careful not to touch the tip before you use it.
8. Shake your hand and "milk" blood down to the end of the finger you are going to prick.
9. Prick the end of your finger and immediately place a drop of blood beside each drop of antiserum. Do not touch the antisera with your finger. It is okay to have someone else prick your finger, but make sure he or she wears protective gloves.
10. Discard the lancet in the sharps container.
11. Put an adhesive bandage on your wound.
12. Using a circular motion, mix each set of drops with a toothpick. Be sure to *use a different toothpick* for each antiserum. Dispose of the toothpicks in the sharps container.
13. Gently rock the slides back and forth for a few minutes or until agglutination occurs.
14. After the agglutination reaction is complete, record the results in the table provided on the data sheet, page 769. Compare your results with the possible results in Table 11-2 to determine your blood type.
15. Collect class data and record these in the table provided on the data sheet. Compare class data with U.S. population data in Table 11-3.



11.22 Prepare the Slides ◆ (A) Draw two circles on a slide and label them "A" and "B" for the type of antiserum each will receive. (B) Draw one circle on a second slide and label it "Rh."

References

American Red Cross. http://www.redcrossblood.org

Lodish, Harvey, Arnold Berk, Paul Matsudaira, Chris A. Kaiser, Matthew P. Scott, S. Lawrence Zipursky, and James S. Darnell. Pages 161–162 in *Molecular Cell Biology*, 5th ed. New York: W. H. Freeman, 2004.

Nelson, David L. and Michael M. Cox. Pages 353–354 in *Lehninger's Principles of Biochemistry*, 4th ed. New York: W. H. Freeman, 2005.

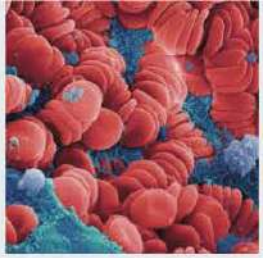
Ross, Michael H. and Wojciech Pawlina. Pages 273–274 in *Histology A Text and Atlas*, 6th ed. Philadelphia: Wolters Kluwer, 2011.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 11-5

Blood Typing

OBSERVATIONS AND INTERPRETATIONS

1 Record your results below.

Antiserum	Agglutination (+/−)
Anti-A	
Anti-B	
Anti-Rh	

My blood type is: _____

2 Record class data in the table below.

Blood Type	Percentage in U.S. Population	Number in Class	Percentage in Class	Deviation from National Values
O+	37.4			
O−	6.6			
A+	35.7			
A−	6.3			
B+	8.5			
B−	1.5			
AB+	3.4			
AB−	0.6			

Source: Stanford School of Medicine. Copyright 2014.

QUESTIONS

1 *Examine the blood type data obtained from your class. Attempt to explain any deviations from the national values.*

2 *People with type "O" are said to be "universal donors" in blood transfusions. Explain the reasoning behind this. Which blood type is designated as the "universal recipient"?*

3 *What biological fact makes the concepts of "universal donor" and "universal recipient" misnomers?*

4 *Maternal-fetal Rh incompatibility (where the Rh− mother's anti-Rh antibodies destroy the fetus's Rh+ red blood cells) is a well-known phenomenon. Less well known are situations of maternal-fetal ABO incompatibility. Suggest combinations of maternal and fetal blood types that could lead to this situation.*

5 *Why are red blood cells used in many indirect agglutination tests?*

ELISA for Detecting Antibodies in a Patient's Sample[1] (The Antibody Capture Method)

EXERCISE 11-6

Theory

ELISA is an acronym for **E**nzyme **L**inked **I**mmuno**s**orbant **A**ssay. As with other serological tests, ELISAs can be used to detect antigen or antibody in a sample. If antigen in a sample is to be identified, you must start with pure antibody to that antigen (homologous antibody). If the goal is to identify antibody in the sample, you must start with the homologous antigen. In either case, a secondary antibody with an attached, or **conjugated**, enzyme as an indicator of antigen-antibody reaction is used.

In this exercise, you will be looking for the presence of antibody in a sample made to simulate patient's serum. Follow along with Figure 11.23 (pages 773–775) as the general process is described. In this version of ELISA, the **purified antigen** (PA) homologous to the antibody being tested for is added to the wells of a microtiter strip. The antigen will **adsorb** (attach) to the plastic wall of the wells. After a wash with buffer to remove unbound antigen, a **blocking agent** (such as gelatin or other protein) may be used to coat the well where antigens have not attached. This prevents antibodies used in subsequent steps from attaching to the well. Following another wash step, the "patient's serum" is added. If the antibodies are present, they will bind specifically to the purified antigen coating the well. Another wash step removes unbound antibody and equally importantly, other components in the patient's serum.

At this point, the test is either positive or negative, but we cannot see it. What is required is an indicator, and that is where the enzyme-linked (**secondary** or **conjugate**) antibody comes in. This antibody is an **immunoglobin antibody**—its antigen is actually an antibody! The first step in the indicator reaction is adding secondary antibodies to the wells. They will bind to the patient's antibodies in the sample, if present. After allowing time for the secondary antibodies to react with their antigen (the patient's antibody), the well is washed again with buffer to remove any unbound secondary antibodies.

The second step in the indicator reaction is to use the enzyme on the secondary antibody to indicate its presence. The specific enzyme chosen for use in an ELISA is only important in that it catalyzes a reaction that can be detected easily. A commonly used enzyme is **horseradish peroxidase** (HRP). HRP catalyzes the conversion of hydrogen peroxide to water and oxygen ($2H_2O_2 \rightarrow O_2 + 2H_2O$). In this exercise, the hydrogen peroxide substrate is associated with a **chromogenic cosubstrate** (3,3',5,5'–tetramethyl-benzidene–TMB). It turns a blue color when it becomes oxidized by the O_2 produced by peroxidase activity, indicating a positive result; a negative result shows no color change because the secondary antibody is not present and the peroxide is not broken down (Fig. 11.24).

Application

ELISA may be used to detect the presence and amount of either antigen or antibody in a sample. In an antibody capture ELISA, the test is used to screen patients for the presence of HIV antibodies (4–8 weeks after exposure), rubella virus antibodies, West Nile virus antibodies, and others. An antigen capture ELISA is used to detect hormones (such as HCG in some pregnancy tests and LH in ovulation tests), drugs, and viral (e.g., HIV p24 antigen within 4–8 weeks after exposure) and bacterial antigens.

In This Exercise

You will perform an ELISA to determine presence of an antibody in a "patient's serum" sample.

✓ Materials

Per Student Group

(The kit is designed for 12 groups of 4 students each)

- ☐ 4 "patient serum" test samples, each with 250 µL (one per student, with or without primary antibody) (yellow tubes, numbered from 1 to up to 48, depending on class size)
- ☐ 1 purified antigen containing 1.5 mL (green microtube—labeled "PA")
- ☐ 1 positive control containing 500 µL (violet microtube—labeled "+")

[1] Bio-Rad ELISA Immuno Explorer™ Kit (Catalog #166-2400EDU), *Bio-technology Explorer™ Instruction Manual*, Rev. D. Bio-Rad Laboratories, Life Science Education. 1-800-4-BIORAD (800-424-6723), www.explorer.bio-rad.com.

- ☐ 1 negative control containing 500 µL (blue microtube—labeled " – ")
- ☐ 1 secondary antibody containing 1.5 mL (orange microtube—labeled "SA")
- ☐ 1 substrate containing 1.5 mL (brown microtube—labeled "SUB")
- ☐ (Optional) 1 microtube of 1% blocking agent containing 1.5 mL—labeled "BA"[2]
- ☐ 2 microtiter plate strips (one per pair of students)
- ☐ 2 (better with 4) micropipettors capable of dispensing 50 µL and tips
- ☐ 4 disposable transfer pipettes
- ☐ 2 beakers of 33 mL wash buffer labeled "WB" (one per pair of students)[3]
- ☐ Large stack of paper towels
- ☐ 2 (up to 4) black marking pens
- ☐ Microtube rack
- ☐ 2 receptacles (plastic cup or something similar—some use a sharps container for this) for pipette tip disposal labeled "tip disposal"

Procedure

Obtain all materials listed (Fig. 11.25). From the yellow microtubes, choose a patient's "serum sample" to test. Record its number on the data sheet, page 777. Refer to the procedural diagram in Figure 11.23 as you read and perform this procedure. Dispose of pipettor tips in the container designated for them.

Step 1 Labeling the Microtiter Strip

With a permanent marker, label the sides of one 12-well microtiter plate strip per student pair as follows: The first three wells with " + ", the second three wells with " – ", the third three wells with one lab partner's sample number, and the fourth three wells with the other lab partner's sample number.

Step 2 Adding Purified Antigen

Using a digital pipettor with a fresh tip, dispense 50 µL of purified antigen (green tube labeled "PA") to each of the wells. Incubate at your desk for 5 minutes. Dispose of the pipette tip properly.

Step 3 Removing Unbound Purified Antigen

Invert the strip and place it on the stack of paper towels (or a paper towel folded several times). Gently tap out the liquids onto the paper towel to remove unbound purified antigen. Avoid contaminating wells with "drainage" from other wells on the towel. Always begin with a fresh towel and replace it if it becomes so wet that there is the potential of contaminating wells with the "drainage."

Steps 4 and 5 Washing the Wells

Fill (but do not overflow) each well with wash buffer using a disposable pipette (which can be used for all subsequent wash steps). Remove the wash buffer onto fresh paper towels. Wash the wells a second time.

Steps 6, 7, 8, and 9 Optional: Using a Blocking Agent

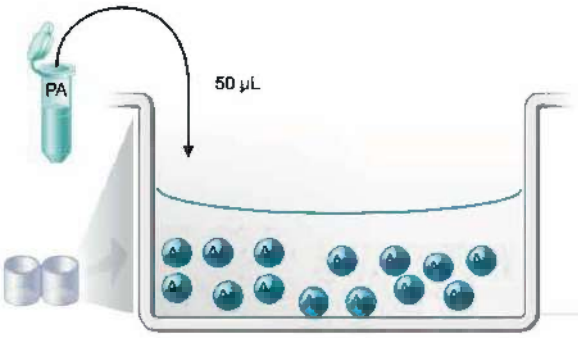
Using a new tip, add 50 µL of blocking agent to each well. Incubate at your lab desk for 5 minutes. Remove unbound blocking agent and wash twice.

Step 1: Labeling the Microtiter Strip Using a permanent marker, label the outside of each well as shown. Replace numbers 1 and 2 with the "Patient Sample" numbers you have been assigned.

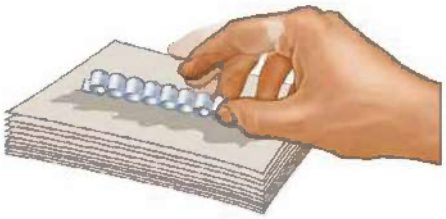
11.23 Procedural Diagram for the ELISA ◆ Pay special attention to volumes, solutions, and pipettes used in each step. Fresh pipette tips must be used for each solution and disposed of properly. The same disposable pipette should be used for transferring wash buffer throughout. Take care not to mix solutions from neighboring wells by overfilling or by emptying over a contaminated part of the paper towels. *(Continues)*

[2] Using blocking agent is an optional step in this protocol. Tubes for this are not supplied in the kit, so must come from instructor's stock. Blocking agent is 1% w/v gelatin, albumin, or nonfat dry milk.

[3] If the blocking step is used, 33 mL of wash buffer is just enough to carry out all the wash steps, but do not waste any! If it is not used, then another 5 mL may be added to the beaker. This presumes the prep is for the maximum 12 student groups of four.

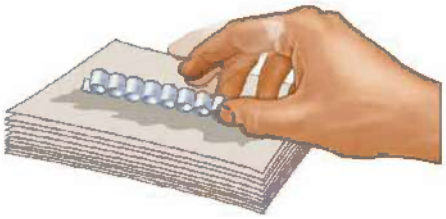


Step 2: Adding Purified Antigen (Ag) Add 50 µL of purified antigen (green microtube labled "PA") to each of the 12 wells. Incubate at your lab desk for 5 minutes. Dispose of the pipette tip properly.

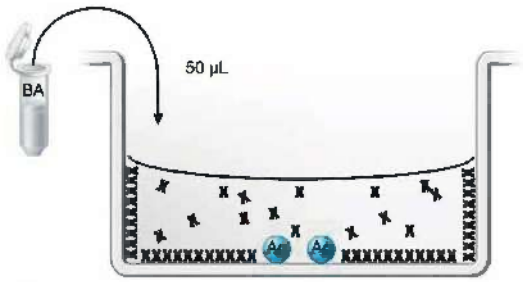


Step 3: Remove Unbound Purified Antigen Gently tap the strip on several thicknesses of fresh paper towels to remove unbound antigen. Be careful not to mix contents between wells. Dispose of wet towels.

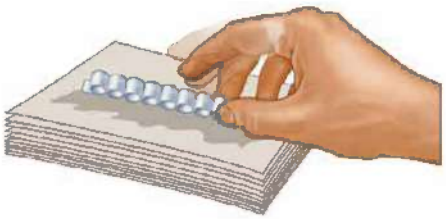
Step 4: Washing the Wells Using the disposable transfer pipette, fill (but don't overfill and mix neighboring well contents) all 12 wells with wash buffer. Save the disposable pipette for subsequent wash steps. Dispose of wet towels. (***Note:*** In reality, at this point the entire lining of the well would be coated with Ag, but only two are shown to reduce cluttering in the art.)



Step 5: Removing the Wash Buffer Drain the wells onto several thicknesses of fresh paper towels, and then gently tap out the remainder. Do not allow mixing between wells. Dispose of wet towels. **Repeat steps 4 and 5 to wash a second time.** Continue with step 6 (optional) or step 10.



Step 6: Adding Blocking Agent (Optional) Add 50 µL of blocking agent (shown as x's in the drawing) to each well. Incubate at your lab desk for 5 minutes.



Step 7: Removing Unbound Blocking Agent Gently tap the strip on several thicknesses of fresh paper towels to remove unbound blocking agent. Be careful not to mix contents between wells.

Step 8: Washing the Wells Using the disposable transfer pipette, fill (but don't overfill and mix neighboring well contents) all 12 wells with wash buffer.

Step 9: Removing the Wash Buffer Drain the wells onto several thicknesses of fresh paper towels. Do not allow mixing between wells. Tap out the last remnants of wash buffer. Dispose of wet towels. **Repeat steps 8 and 9 to wash a second time.**

11.23 Procedural Diagram for the ELISA *(Continues)* ♦

Step 10 Adding the Positive Control

Using the digital pipettor, add 50 µL of positive control (violet tube labeled "+") to each of the wells labeled "+". Dispose of the tip in the designated container. Continue immediately with step 11.

Step 11 Adding the Negative Control

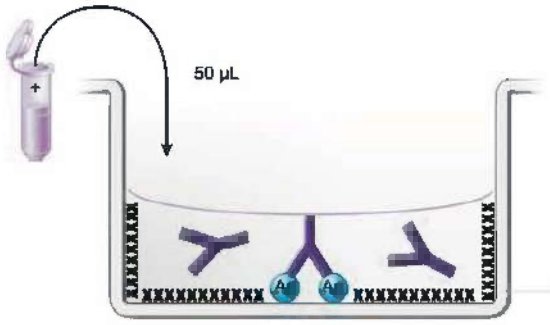
Using a new tip, add 50 µL of negative control (blue tube labeled "−") to each of the wells labeled "−". Dispose of the tip in the designated container. Continue immediately with Step 12.

Step 12 Adding Patient "Serum Samples"

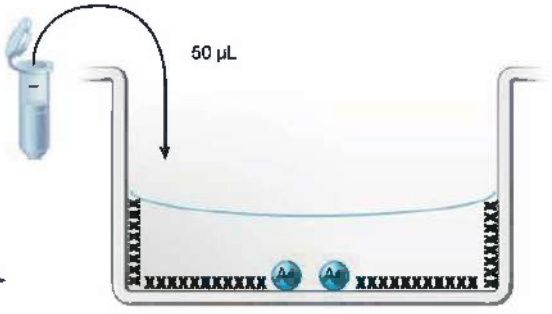
Using a new tip, add 50 µl of one sample (yellow microtube) to the wells marked with its number. Dispose of the tip in the designated container. Using a new tip, add 50 µl of the second sample (yellow microtube) to the wells marked with its number. Dispose of the tip in the designated container. Incubate at your lab desk for 5 minutes.

Step 13 Removing the Control and Patient's "Serum Sample" Solutions and Washing the Wells

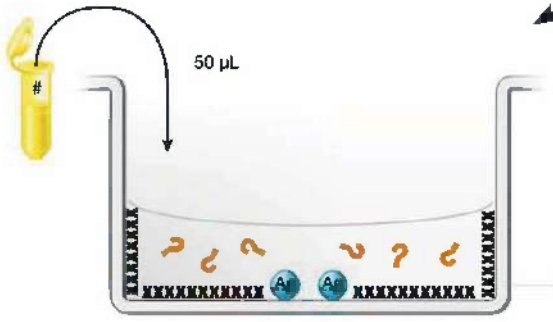
Drain the controls and patient samples out of the wells onto a fresh paper towel and gently tap out any residual as in step 3. Be careful not to mix solutions between wells. Wash the wells as in steps 8 and 9, twice.



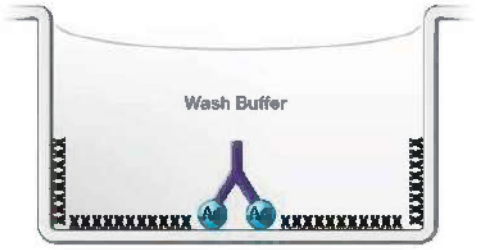
Step 10: Adding the Positive Control Add 50 µL of positive control (violet microtube labeled "+") **to the three "+" wells.** Continue immediately with step 11.



Step 11: Adding the Negative Control Add 50 µL of negative control (blue microtube labeled "−") **to the three "−" wells.** Continue immediately with step 12.



Step 12: Adding Patient "Serum Samples" Add 50 µL of "Patient Serum Samples" (yellow microtubes labeled with numbers) to the appropriate remaining wells. Incubate for 5 minutes at your lab desk. The question marks indicate that the contents of the Patient's Sample are unknown.



Step 13: Removing the Control and Patient "Serum Sample" Solutions and Washing the Wells Gently tap the strip on several thicknesses of fresh paper towels to remove the Control and "Patient Serum Sample" solutions. Be careful not to mix contents between wells. **Perform two washes with wash buffer as shown in steps 8 and 9.** The illustration shows what a positive result looks like at this point. A negative result would not have the antibody attached to the antigens and would look like the art in step 11.

11

11.23 Procedural Diagram for the ELISA *(Continues)* ♦

Step 14 Adding Secondary (Conjugate) Antibody

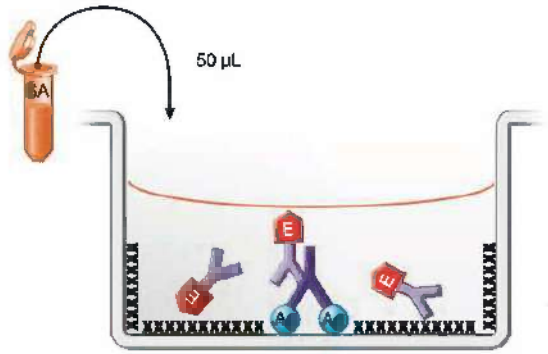
Using a new tip, dispense 50 µL of secondary antibody (orange microtube labeled "SA") to each of the wells. Incubate at your desk for 5 minutes.

Step 15 Removing Unbound Secondary Antibody

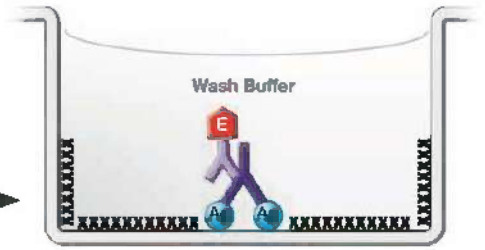
Remove the secondary antibody by gently tapping the strip on fresh towels as in step 3, and then wash three times as in steps 8 and 9. Replace towels as necessary.

Step 16 Adding Substrate

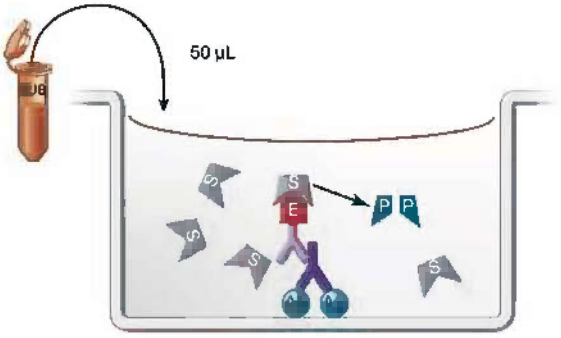
Using a new tip, dispense 50 µL of substrate (brown tube labeled "SUB") to all 12 wells. Incubate at your lab desk for 5 minutes. Observe for color changes and record your results on the data sheet, page 777. Take your reading within 5 minutes because there is the potential for a blue color developing (due to a reaction with light) if allowed to incubate longer.



Step 14: Adding Secondary (Conjugate) Antibody Add 50 µL of secondary antibody (orange microtubes labeled with "SA") to the 12 wells. Incubate for 5 minutes at your lab desk.



Step 15: Removing Unbound Secondary Antibody Gently tap the strip on several thicknesses of fresh paper towels to remove unbound secondary antibody solution. Be careful not to mix contents between wells. **Perform three washes with wash buffer as shown in steps 8 and 9.** The illustration shows what a positive result looks like at this point. A negative result would not have either antibody present and would look like the art in step 11.



Step 16: Adding Substrate Add 50 µL of substrate (brown microtube labeled "SUB") to the 12 wells. Incubate at your lab desk and read results within 5 minutes. A blue color is a positive result. **Waiting longer than 5 minutes to read results could lead to the substrate turning blue through its exposure to light, not enzyme.**

11.23 Procedural Diagram for the ELISA *(Continued)* ♦

11.24 Positive and Negative Results ◆ A positive result in this ELISA produces a blue color within 5 minutes; no reaction is colorless. Waiting longer than 5 minutes may lead to a nonspecific (that is, not due to enzyme activity) color change.

11.25 Materials Needed ◆ Shown are the materials needed for this procedure, but not necessarily in the proper quantity (due to space limitations). The tubes in the rack are as follows: yellow are numbered "patient serum" samples, green is purified antigen ("PA"), violet is the positive control ("+"), blue is the negative control ("–"), orange is the secondary antibody ("SA"), brown is the substrate ("SUB"), and clear is the optional 1% blocking agent ("BA"). Towels, a disposable transfer pipette, a permanent marker, wash buffer (WB), digital pipettor and tips, can for tip disposal, and microtiter strip are also shown.

References

Bio-Rad ELISA Immuno Explorer™ Kit (Catalog #166-2400EDU), *Bio-technology Explorer™ Instruction Manual*, Rev. D. Bio-Rad Laboratories, Life Science Education. 1-800-4-BIORAD (800-424-6723), www.explorer.bio-rad.com.

Lam, Joseph S. and Lucy M. Mutharia. Chap. 5 in *Methods for General and Molecular Bacteriology*. Philipp Gerhardt, R.G.E. Murray, Willis A. Wood, and Noel. R. Krieg, eds. Washington, DC: ASM Press, 1994.

Madigan, Michael T., John M. Martinko, Paul V. Dunlop, and David P. Clark. Pages 922–926 in *Brock's Biology of Microorganisms*, 12th ed. San Francisco: Pearson/Benjamin Cummings, 2009.

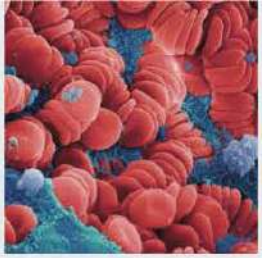
Shores, Teri. Pages 102–103 in *Understanding Viruses*. Sudbury, MA: Jones and Bartlett Publishers, 2009.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 11-6

ELISA for Detecting Antibodies in a Patient's Sample (The Antibody Capture Method)

Take your readings within 5 minutes because there is the potential for a blue color developing (due to a reaction with light) if allowed to incubate longer.

OBSERVATIONS AND INTERPRETATIONS

1 Record your Patient's Sample Number _____.

2 Write the number of yours and your partner's Patient Sample Numbers in the table below.

Tube labels	+	+	+	–	–	–						
Results												

3 Record the results in each well of the microtiter strip in the table above.

QUESTIONS

1 *Describe the expected consequences of the following procedural errors,*

a. *Mixing the positive control solution in a negative control well during step 13.*

b. *Mixing the negative control solution in a positive control well during step 13.*

c. *Mixing primary antibody in a negative control well after step 13.*

d. *Mixing secondary antibody with negative control after step 15.*

2 *Consider the positive controls.*

a. *Given the design of this ELISA, what is the essential component in the positive control solution? Choose from antigen, primary antibody, secondary antibody, and substrate.*

b. *What purpose do the positive controls serve?*

c. *What are possible causes of negative results in all the positive controls?*

d. *How would negative results in* all *the positive controls affect your interpretation of results given by the patient samples?*

3 *Consider the negative controls.*

a. *Given the design of this ELISA, what component must be* absent *in the negative control solution? Choose from antigen, primary antibody, secondary antibody, and substrate.*

b. *What purpose do the negative controls serve?*

c. *What are possible causes of positive results in all the negative controls?*

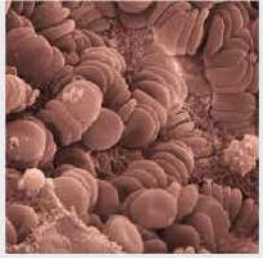
d. *How would positive results in all the negative controls affect your interpretation of results given by the patient samples?*

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____



DATA SHEET 11-6

(continued)

4 *Consider the samples tested.*

a. *Why are the samples run in triplicate?*

b. *What might explain inconsistent results within each triplicate set and how might they affect your interpretations of the patient samples?*

1) Positive controls

2) Negative controls

3) Patient sample

5 *Refer to the procedural diagram on pages 772–775 for the following questions:*

a. *In Step 4, what could be the consequence of washing too vigorously with wash buffer? Of not washing enough?*

b. *What might be the consequence of not adding blocking agent in step 6? (Omit this question if blocking agent was not used.)*

c. *In step 13, what would be the consequence of washing too vigorously with wash buffer? Of not washing enough?*

d. *In step 15, what would be the consequence of washing too vigorously with wash buffer? Of not washing enough?*

SECTION 12

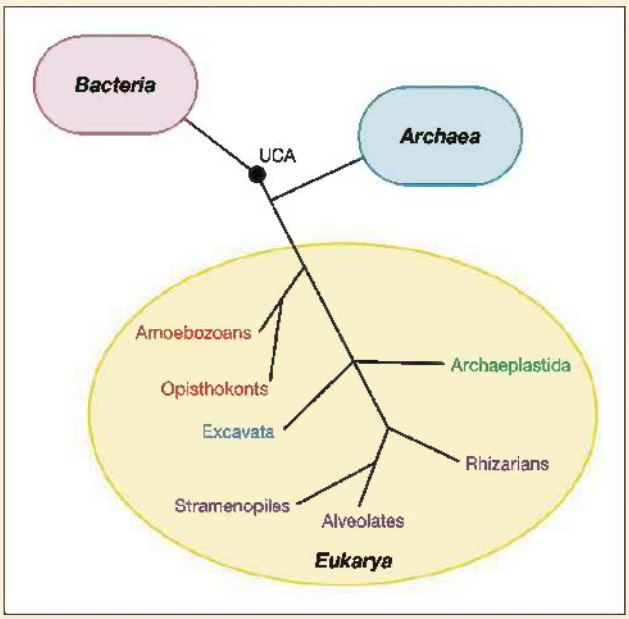
Eukaryotic Microbes

Bacteriology dominates most college microbiology courses, but the discipline of microbiology also includes eukaryotic microscopic organisms. You had the opportunity to observe some simple eukaryotic microorganisms in Exercises 3-3 and 3-4. In this section, you will examine some representative fungi of medical or economic importance (Exercise 12-1) and learn a method of culturing them for microscopic observation (Exercise 12-2). These are followed by a survey of medically important simple eukaryotes (Exercise 12-3). Lastly, Exercise 12-4 introduces you to parasitic helminths (worms), because their identification is often the responsibility of microbiologists who examine patient samples. Thus, these animal parasites have entered the domain of microbiology.

Eukaryotic Supergroups—A Refresher

Current evidence suggests that the members of Domain *Eukarya* cluster into four supergroups (Fig. 12.1). These are: Excavata, SAR, Archaeplastida, and Unikonta. The SAR supergroup is a combination of two formerly recognized supergroups (Chromalveolata and Rhizaria) and gets its acronym from the names of three major lineages within it: **S**tramenopiles, **A**lveolates, and **R**hizarians. Because of the number of species in each, it should not be surprising that supergroups exhibit great diversity. It also should not be surprising that this overhaul of Domain *Eukarya* is a work in progress and will undergo further revision as new relationships are discovered.

- Excavates are unicellular flagellates with a feeding groove *excavated* from one side of the cell. Within this group are species with atypical mitochondria and flagella. Excavates (e.g., *Euglena*) were covered in Exercises 3-3 and 3-4. Pathogenic examples will be covered in Exercise 12-3.
- SAR stands for **S**tramenopile, **A**lveolate, **R**hizaria. Stramenopiles are photosynthetic organisms (e.g., diatoms) and were covered in Exercises 3-3 and 3-4. Membrane-bound sacs located next to the plasma membrane characterize the alveolates, which include both autotrophic and heterotrophic species, some of which also were seen in Exercises 3-3 and 3-4 (e.g., *Paramecium*). Some pathogenic examples will be examined in Exercise 12-3. Rhizarians (discussed in Exercise 3-4) are not covered in this section, but they are unicellular autotrophs and heterotrophs characterized by threadlike pseudopodia (cytoplasmic extensions) used for locomotion and/or feeding. Examples include foraminifera and radiolarians.
- Archaeplastids appear to have descended from a common ancestor that engulfed a cyanobacterium that eventually evolved into the chloroplast by primary endosymbiosis. They were covered in Exercises 3-3 and 3-4 (e.g., *Volvox* and *Chlamydomonas*). This group also includes true plants.
- Unikonta is subdivided into two main groups: Amoebozoans, which have lobe-shaped pseudopods, and opisthokonts, which have posterior flagella and includes animals, fungi, and their closest unicellular relatives. Pathogenic fungi are covered in Exercises 12-1 and 12-2, and pathogenic animals (parasitic worms) are covered in Exercise 12-4. ◆



12.1 Domain Eukarya and Its Supergroups ◆
There is provisional evidence that Unikonta diverged from the common ancestor of the other supergroups and is therefore shown branching first, but this is not a certainty. There is no clear evidence as to the order of divergence between the remaining three supergroups, so they are shown as branching from a single node. Amoebozoan, opisthokont, excavate, and alveolate pathogens are discussed in this section. Color key: Unikonta–red; Excavata–blue; Archaeplastida–green; SAR–violet.

Representative Fungi of Medical and Economic Importance

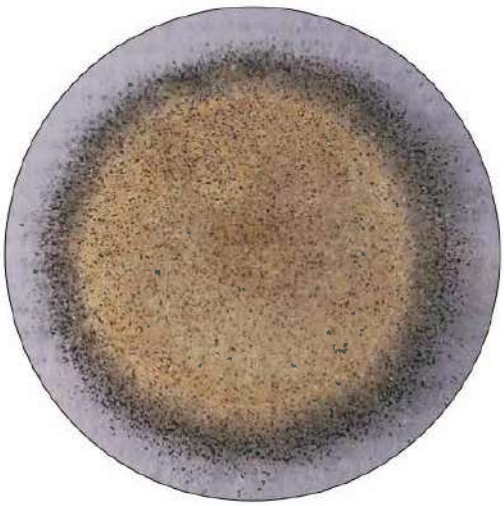
EXERCISE 12-1

Theory

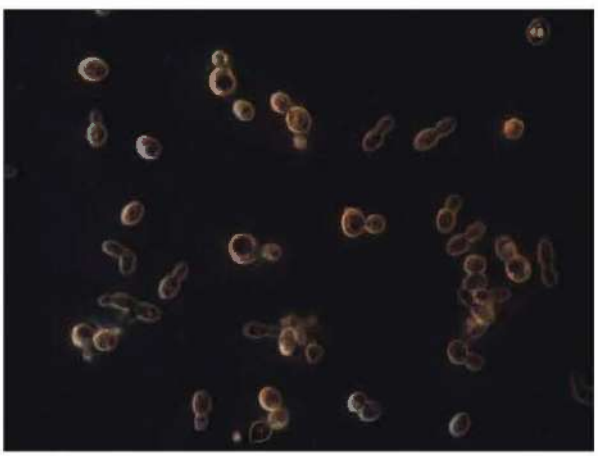
Current evidence suggests that members of the eukaryotic kingdoms Fungi and Animalia are closely related (as kingdoms go) and consequently they have been placed into the informal taxonomic group Opisthokonta within the eukaryotic supergroup Unikonta, which refers to their possession of posterior flagella (in contrast to other eukaryotes that have anterior flagella). Evidence also suggests that their common ancestor, last shared between 1 billion and 1.5 billion years ago, was a unicellular flagellate, as are the fungal groups that diverged earliest in their history. However, most extant fungi no longer have flagella and are nonmotile. Fungi are heterotrophic, but unlike animals (that ingest, then digest their food), fungi are **absorptive heterotrophs**; that is, they secrete exoenzymes into the environment, and then absorb the digested nutrients. Their cell wall is usually made of the polysaccharide **chitin**, not cellulose as in plants. Most are **saprophytes** that decompose dead organic matter, but some are **parasites** of plants, animals, or humans.

Fungi are informally divided into unicellular **yeasts** (Fig. 12.2) and filamentous **molds** (Fig. 12.3) based on their overall appearance. **Dimorphic fungi** have both mold and yeast life-cycle stages. Filamentous fungi that produce fleshy reproductive structures—mushrooms, puffballs, and shelf fungi—are referred to as **macrofungi** (Fig. 12.4), even though the majority of the fungus is filamentous and hidden underground or within decaying matter.

Individual fungal filaments are called **hyphae**, and collectively they form a **mycelium**. The hyphae are darkly pigmented in **dematiaceous fungi** and unpigmented in **hyaline** or **moniliaceous fungi** (Fig. 12.5). Hyphae may be **septate**, in which walls separate adjacent cells, or **nonseptate** if walls are absent (Fig. 12.6).



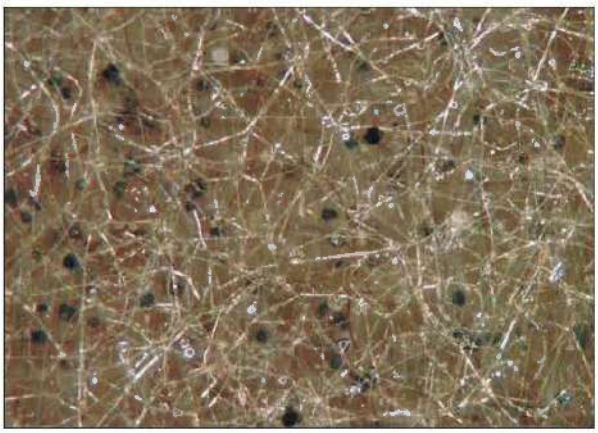
12.3 Mold ◆ Molds grow as fuzzy colonies. Their spores are abundant in the environment and frequently contaminate agar plates.



12.2 Yeast ◆ The brewer's yeast *Saccharomyces cerevisiae* is shown in a wet mount preparation using phase contrast microscopy. The cells are oval with dimensions of 3 µm–8 µm by 5 µm–10 µm. Short chains of cells (pseudohyphae) are visible in this field.

12.4 Macrofungus ◆ This mushroom is the fruiting body from a mycelium that is busily engaged in decomposing the redwood log below it.

Fungal life cycles usually are complex, involving both sexual and asexual forms of reproduction. Gametes are produced by **gametangia** and spores are produced by a variety of **sporangia.** While gametes and spores are both reproductive cells, they function differently in advancing the life cycle. A spore does so simply by dividing (asexual reproduction), whereas gametes, which are haploid, need to combine by fertilization and produce a zygote with a single diploid nucleus (sexual reproduction). The familiar situation is that fusion of cytoplasm (plasmogamy) and joining of nuclei (karyogamy) happen one right after the other. Not so in fungi! Karyogamy is usually delayed following plasmogamy, resulting in a **dikaryotic** cell with two separate haploid nuclei. This cell usually divides repeatedly to produce dikaryotic hyphae. At some point karyogamy occurs and a diploid zygote is formed, which undergoes meiosis to produce haploid sexual spores ("sexual" because they are part of the sexual life cycle). So, unlike animals, the only diploid cell in most fungal life cycles is the zygote, and the spores it produces are characteristic of the specific fungal group.

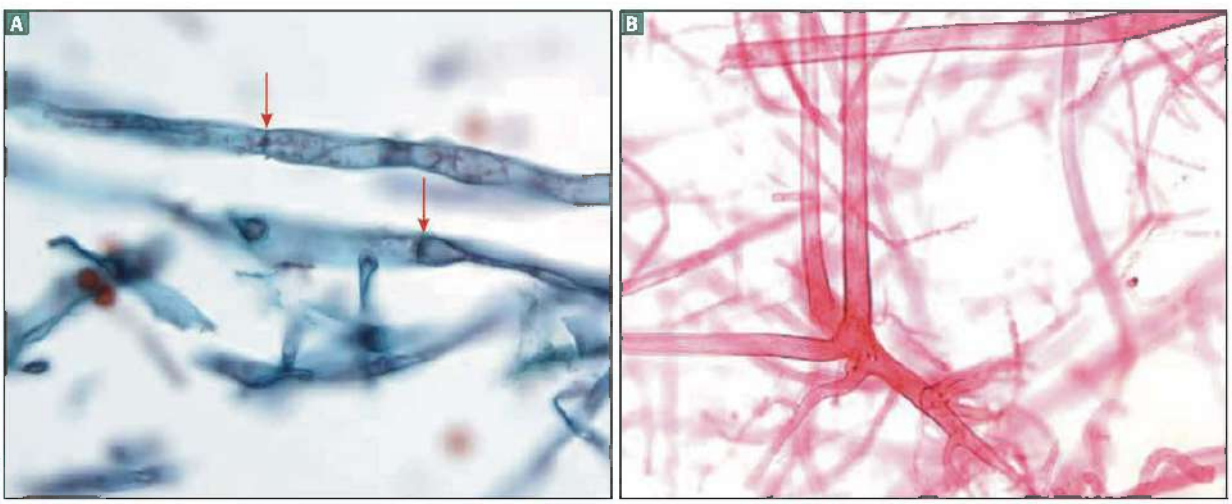


12.5 Hyaline Hyphae ◆ This tangled mass of hyphae belongs to the bread mold, *Rhizopus.* They are hyaline, because they lack pigment.

Many fungi also produce characteristic asexual spores during their life cycle. If they form at the ends of hyphae, they are called **conidia.** Other asexual spores are **blastospores** (blastoconidia), which are produced by budding, and **arthrospores**, which are produced when a hypha breaks. **Chlamydospores** (**chlamydoconidia**) are formed at the end of some hyphae and are a resting stage.

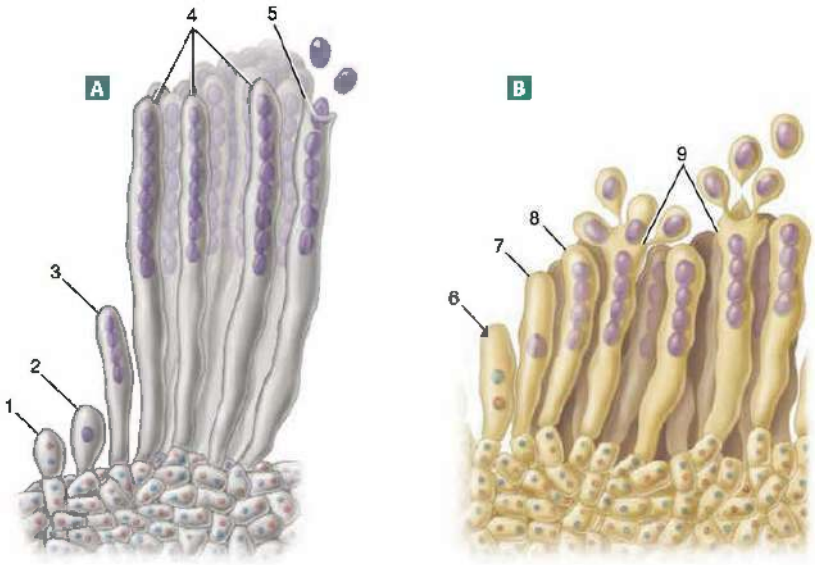
The fungus kingdom is divided into divisions (phyla) primarily based on the pattern of sexual spore production and the presence of septa in the hyphae. Members of the **division Zygomycota** are terrestrial, have nonseptate hyphae, and form **zygospores** when the zygote undergoes meiosis. They also produce asexual **sporangiospores.** Members of the **division Ascomycota** (formerly **class Ascomycetes**) produce an **ascus** (sac) in which the zygote undergoes meiosis to produce haploid **ascospores** (Fig. 12.7A). Ascomycete hyphae are septate. This group also includes yeasts, which are usually unicellular. Members of the **division Basidiomycota** (formerly **class Basidiomycetes**) have septate hyphae and during sexual reproduction produce a **basidium** that undergoes meiosis to produce four **basidiospores** attached to its surface (Fig. 12.7B). Both ascomycetes and basidiomycetes produce asexual conidia.

Following is a brief survey of fungi that are likely to be encountered in an introductory microbiology class and selected medically important fungi.



12.6 Septate and Nonseptate Hyphae ◆ **(A)** The hyphae of *Aspergillus* are septate. Note the crosswalls (designated by arrows) dividing cells. **(B)** *Rhizopus* provides an example of nonseptate hyphae.

12.7 Sexual Sporangia ◆ Ascomycota and basidiomycota are major fungal divisions distinguished by their sexual sporangia, in which the zygote undergoes meiosis to produce haploid spores. Because crossing over occurs in meiosis, the resulting spores are genetically different from each other, as are the hyphae that develop from them. (**A**) Shown in this drawing of a magnified ascomycete mycelium are dikaryotic hyphae along the base and asci in various stages of development. Each ascus typically produces four pairs of ascospores, which are released and develop into a new mycelium. Ascomycetes are commonly called sac fungi because their spores develop within the saclike sporangium. Key to Figure Labels: **1** Dikaryotic ascus with two haploid nuclei; **2** Ascus with zygote nucleus following karyogamy; **3** Ascus with four haploid ascospore nuclei following zygotic meiosis; **4** Asci with eight ascospores following mitosis of the four ascospores; **5** Ascus releasing ascospores. (**B**) Basidiomycetes produce their spores at the ends of elongated basidia that resemble a club, hence their common name, club fungi. This drawing illustrates a portion of the dikaryotic mycelium and several basidia. One could find these along the edges of a mushroom's gills. Each haploid nucleus enters an extension of cytoplasm that buds off and is swept away to develop into a new mycelium. Key to Figure Labels: **6** Dikaryotic basidium; **7** Basidium with zygote nucleus following karyogamy; **8** Basidium with four haploid nuclei following zygotic meiosis; **9** Basidiospores being released.

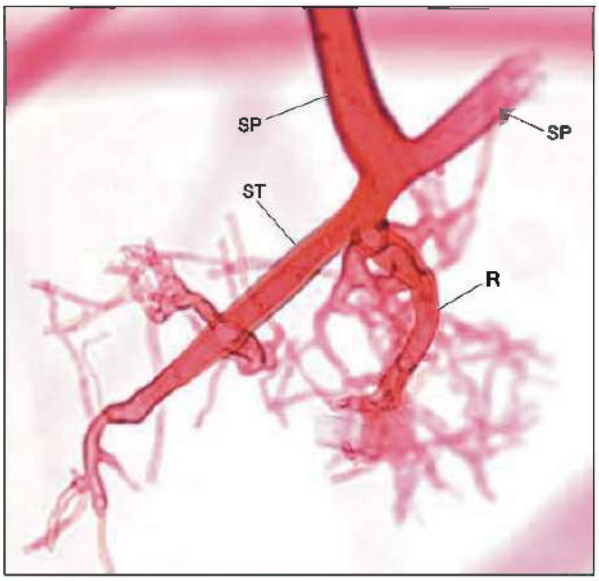


Division Zygomycota

Rhizopus

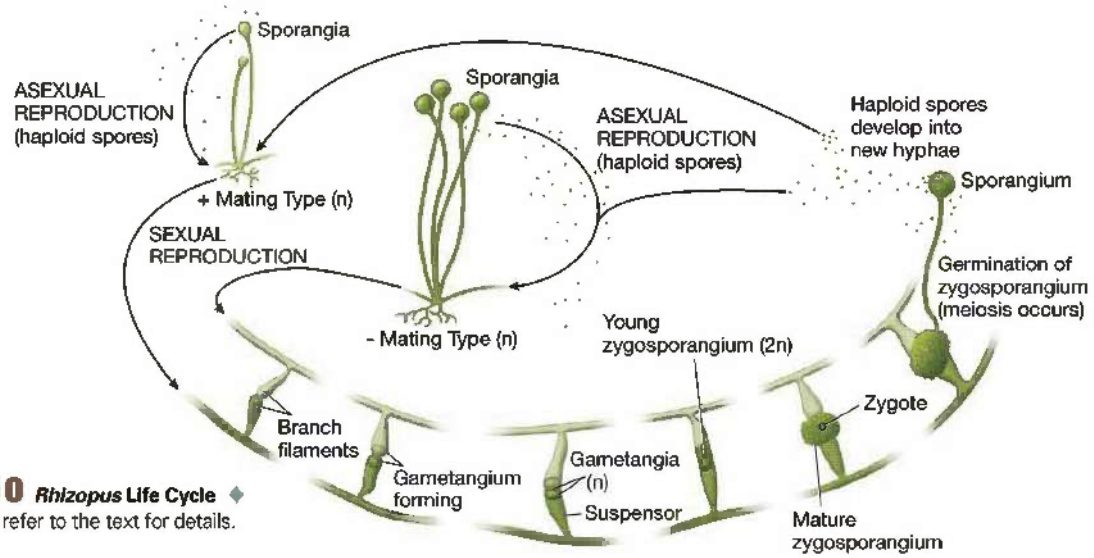
Rhizopus species are naturally found associated with living and decaying plant material, and in the former case can cause spoilage. There are about a dozen species. They are fast-growing molds that produce white or grayish, cottony growth. The mycelium becomes darker with age as sporangia are produced, giving it a "salt and pepper" appearance (Fig. 12.8). Microscopically, *Rhizopus* species produce broad (10 µm), hyaline, and usually nonseptate surface and aerial hyphae with irregular diameters. Anchoring **rhizoids** (Fig. 12.9) are produced where the surface hyphae (**stolons**) join the bases of the long, unbranched **sporangiophores**.

12.8 *Rhizopus stolonifer* Culture ◆ Black asexual sporangia of this bread mold have begun to form at the top, giving the growth a "salt and pepper" appearance.



12.9 *Rhizopus* Rhizoids ◆ Anchoring rhizoids (R) form at the junction of each sporangiophore (SP) and the stolon (ST). Note the absence of the septa.

12

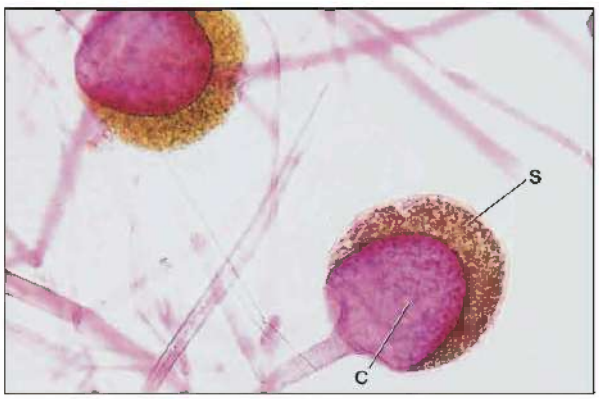


12.10 ***Rhizopus* Life Cycle** ◆
Please refer to the text for details.

The *Rhizopus* life cycle (Fig. 12.10) has both sexual and asexual phases. Asexual sporangiospores are produced by large, circular sporangia (Fig. 12.11) borne at the ends of the elevated sporangiophores. A hemispherical **columella** supports the sporangium. After release, the sporangiospores develop into hyphae identical to those that produced them.

On occasion, sexual reproduction occurs when hyphae of different mating types (designated + and − strains) make contact. Initially, **progametangia** (Fig. 12.12) extend from each hypha and nuclei and cytoplasm flow into them. As a consequence, they enlarge and contact one another. Upon contact, a septum separates the end of each progametangium into a gametangium and a **suspensor** (Fig. 12.13). The walls between the two gametangia dissolve, and a thick-walled **zygosporangium** develops (Figs. 12.14 and 12.15). Fusion of nuclei (karyogamy) occurs within the zygosporangium, resulting in multiple diploid zygote nuclei. After a dormant period, zygote meiosis occurs and produces zygospores. The zygosporangium germinates and produces a sporangium similar to asexual sporangia. Haploid zygospores are released, develop into new hyphae, and the life cycle is completed.

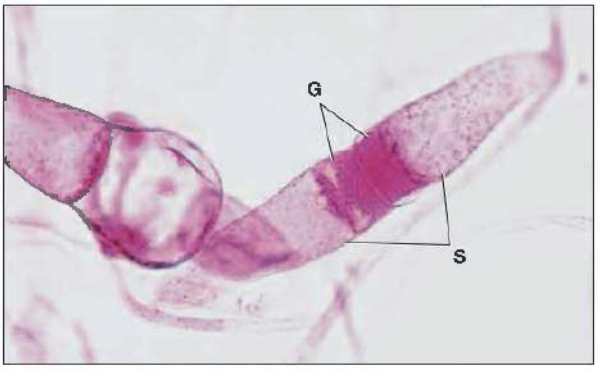
Rhizopus species are common, especially in soil and decaying organic material, and are found worldwide. *R. oryzae* and *R. microsporus* are the two species most responsible for producing **zygomycosis** (mucormycosis), a condition found most frequently in diabetics, organ transplant recipients, and immunocompromised patients. Portal of entry and overall health of the patient are critical factors in determining the location and extent of infection. Sites of infection include the nasal cavity, brain, abdominopelvic cavity, skin, and lungs. Entry into the blood leads to rapid spreading of the organism, occlusion of blood vessels, and necrosis of tissues. Treatment



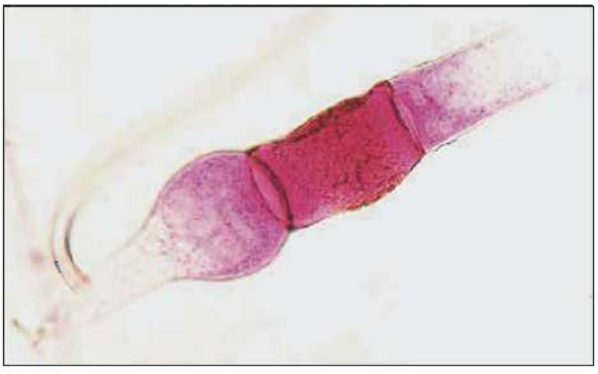
12.11 ***Rhizopus* Sporangiophores** ◆ The sporangium is found at the end of a long, unbranched, and nonseptate sporangiophore. The haploid asexual sporangiospores (S) in the sporangium cover the surface of the columella (C), which has a flattened base.

12.12 ***Rhizopus* Progametangia** ◆ The black circle encloses progametangia (branch filaments) from different hyphae. Contact between them results in each forming a gamete.

with antifungal agents and correcting the cause of susceptibility (e.g., improved nutrition) increase survival, but the mortality rate is high—between 47%–84% worldwide. On a less severe note, *R. stolonifer* is the common bread mold.



12.13 *Rhizopus* Gametangia and Suspensors ◆ Gametangia (G) and suspensors (S) are shown in the center of the field. Gametangia contain one or more haploid nuclei from each mating type.



12.14 Young *Rhizopus* Zygosporangium ◆ The zygosporangium forms when the cytoplasm from the two mating strains fuse (plasmogamy).

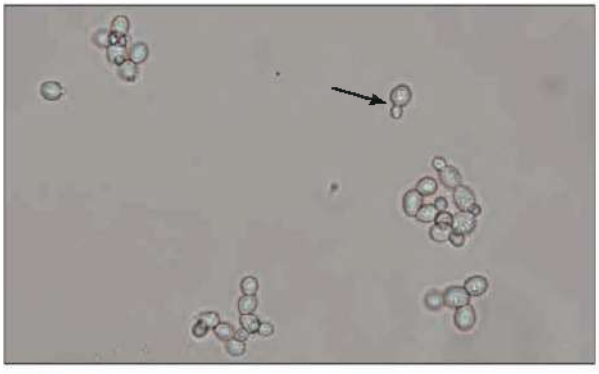
12.15 Mature *Rhizopus* Zygosporangium ◆ Haploid nuclei from each strain fuse within the zygosporangium (karyogamy) to produce many diploid zygote nuclei. Meiosis follows to produce numerous haploid zygospores.

Division Ascomycota

Saccharomyces cerevisiae

Saccharomyces cerevisiae is an ascomycete used in the production of bread, wine, and beer but is not an important human pathogen. It does not form a mycelium but, rather, produces a colony similar to bacteria (Fig. 12.16). Vegetative **blastospores** are generally oval to round in shape, and asexual reproduction occurs by budding (Fig. 12.17). Short **pseudohyphae** are sometimes produced when the budding cells fail to separate. In an *S. cerevisiae* population, haploid and diploid cells are indistinguishable from one another and both reproduce asexually by budding. Diploid cells occasionally undergo meiosis (acting as an ascus) and produce one to four ascospores. When released, ascospores bud to produce more haploid cells. Occasionally, haploid cells of opposite mating types combine to create a new diploid cell, thus acting like gametes.

12.16 *Saccharomyces cerevisiae* Colony ◆ Note that the appearance is similar to a typical bacterial colony, not "fuzzy" like mold colonies.



12.17 *Saccharomyces cerevisiae* ◆ This bright-field micrograph is of an unstained wet mount of the yeast *Saccharomyces cerevisiae*. Notice the budding cell (arrow).

Candida albicans

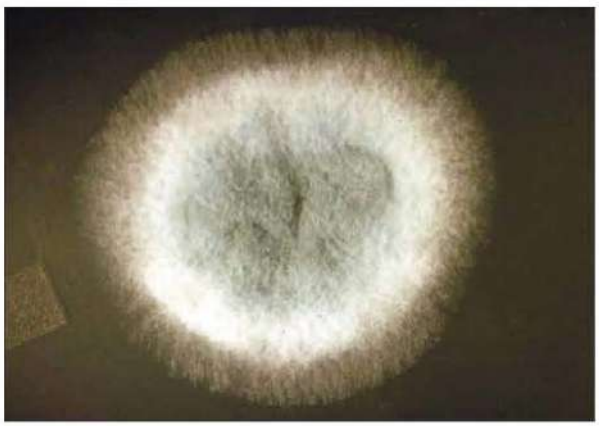
Candida albicans (Fig. 12.18) is part of the normal respiratory, gastrointestinal, and female urogenital tract floras, but it is also the most common fungal opportunistic pathogen. It is the causative agent of thrush in the oral cavity, vulvovaginitis of the female genitals, and cutaneous candidiasis of the skin. Systemic candidiasis may follow infection of the lungs, bronchi, or kidneys. Entry into the blood may result in endocarditis. The ability to attach to epithelial surfaces of mucous membranes and subsequently penetrate to deeper tissues are important virulence factors. Individuals most susceptible to *Candida* infections are diabetics, those with immunodeficiency (e.g., AIDS), catheterized patients, and individuals taking antimicrobial medications. Treatments vary depending on the location of infection and the state of the patient.

Candida albicans cells are round to oval that reproduce by budding and are 3 µm–6 µm in diameter. Sometimes the cells separate after budding, but other times they remain attached, forming septate chains of two types: **pseudohyphae**, which are narrower at their junctions, and true hyphae, which are not. Both types branch and produce asexual blastospores. The ability to switch growth pattern between pseudohyphae and true hyphae may increase virulence. Large, round, thick-walled chlamydospores form at the ends of pseudohyphae. The sexual life cycle is similar to *S. cerevisiae*.

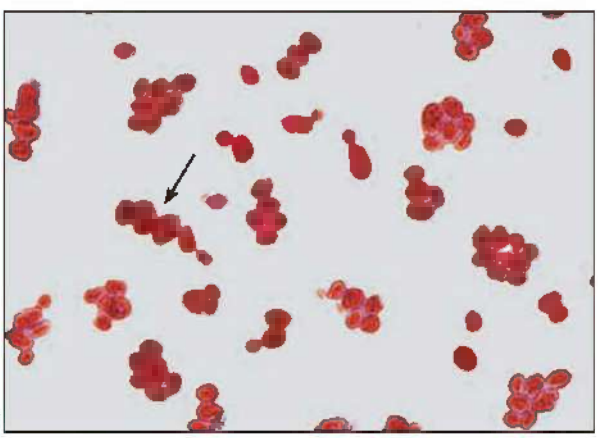
Aspergillus

Aspergillus species are commonly found in soil and on plants. Roughly 180 species have been identified. They are characterized by green to yellow or brown granular colonies with a white edge (Fig. 12.19). One species, *A. niger*, produces distinctive black colonies. Vegetative hyphae are hyaline (unpigmented) and septate. The *Aspergillus* fruiting body is distinctive, with chains of conidia arising from one (uniseriate) or two (biseriate) rows of **phialides** attached to a swollen **vesicle** at the end of an unbranched conidiophore (Fig. 12.20). The conidiophore grows from a foot cell in the vegetative hypha (Fig. 12.21). Fruiting body structure and size, and conidia color are useful in species identification. Since their discovery it was thought that *Aspergillus* species were incapable of sexual reproduction, but evidence is mounting that under the right environmental conditions viable ascospores can be produced.

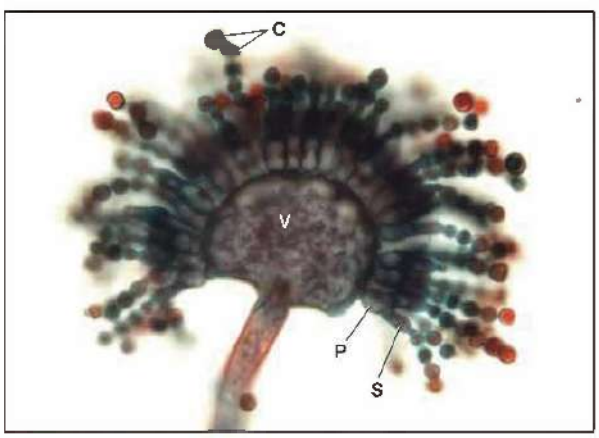
A. fumigatus and other species are opportunistic pathogens that cause aspergillosis, an umbrella term covering many diseases. Immunocompromised patients are at highest risk of infection. Invasive aspergillosis is



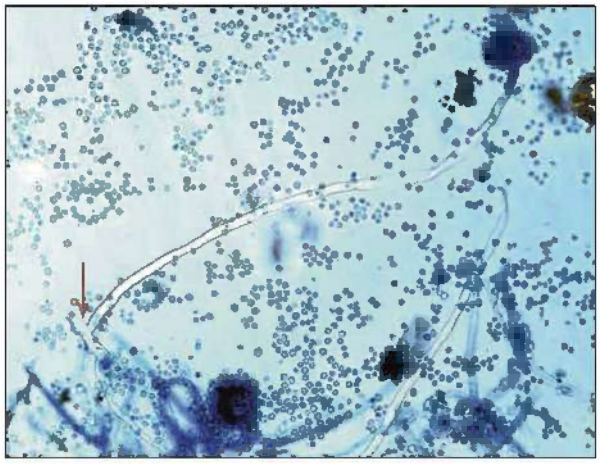
12.19 *Aspergillus fumigatus* Colony on Sabouraud Dextrose Agar ◆ Note the rugose topography and green, granular appearance with a white margin. The reverse is white. Although a common cause of aspergillosis, normally healthy people are not at great risk from *A. fumigatus* infection.



12.18 *Candida albicans* Vegetative Cells Grown in Culture ◆ Cells grown on a solid medium don't show natural arrangements seen in tissue samples. However, in this micrograph the oval cell shape and a pseudohypha (arrow) with a budding cell at its end are visible.



12.20 *Aspergillus* Conidial Head ◆ Shown is a section of an *Aspergillus niger* conidiophore. The conidia (C), primary (P) and secondary (S) phialides, and vesicle (V) are visible. This is a biseriate conidium because of the two phialide layers.



12.21 *Aspergillus* Foot Cell (Lactophenol Cotton Blue Stain) ♦ Sporangiophores emerge from a foot cell with the shape of an inverted "T" (arrow). This is a wet mount of *A. flavus*. Notice all the conidia in the field.

the most severe form and has high mortality. It results in necrotizing pneumonia and may spread to other organs, such as the heart and central nervous system. Antifungal medications can be used in treatment, but identification of the pathogen to species level is necessary because of their different sensitivities. Allergic aspergillosis may occur in individuals who are in frequent contact with the spores and become sensitized to them. Subsequent contact produces symptoms similar to asthma. Aspergilloma (fungus ball) involves colonization of the paranasal sinuses or lungs, resulting in abscess formation. It is frequently asymptomatic and resolves without treatment, but in serious cases it can be fatal.

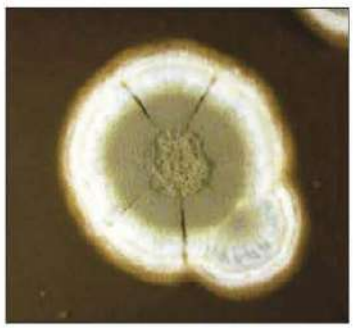
Some *Aspergillus* species are of commercial importance. Fermentation by *A. oryzae* is used in the production of sake from rice and soy paste from soybeans. *A. oryzae* and *A. soyae* may be further fermented into soy sauce. Aspergilli are also used in commercial production of citric acid, various enzymes, and many other products. Further, *A. niger* and *A. oryzae* have been designated as GRAS (generally regarded as safe) by the FDA and WHO, which has opened the door for using them as hosts in biotechnological applications.

Penicillium

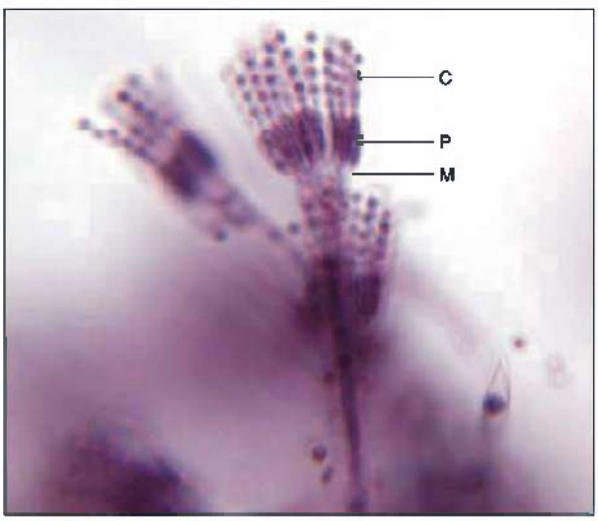
Members of the genus *Penicillium* are ubiquitous, being found in the air, soil, and decaying organic matter worldwide. Over 200 species have been identified. They produce distinctive green, powdery, radially furrowed colonies with a white apron (Fig. 12.22) and light-colored reverse surface. The hyphae are septate and thin. Distinctive *Penicillium* fruiting bodies, consisting of **metulae**, phialides, and chains of spherical conidia, are located at the ends of branched or unbranched conidiophores (Fig. 12.23).

Although not an important feature in laboratory identification, sexual reproduction occurs under the proper conditions and results in the formation of ascospores within an ascus.

Penicillium is best known for its production of the antibiotic penicillin (discovered by Alexander Fleming in 1928), but it is also a common contaminant. One pathogen, *P. marneffei*, is endemic to Asia and is responsible for disseminated opportunistic infections of the lungs, liver, and skin in immunosuppressed and immunocompromised patients. It is thermally dimorphic, producing a typical velvety colony with a distinctive red pigment at 25°C but converting to a yeast form at 35°C. Other species are important, because they cause spoilage of fruits and vegetables. *P. chrysogenum* is common in damp, indoor habitats and their spores can cause allergic reactions. Other species of *Penicillium* are of commercial importance for fermentations used in cheese production. Examples include *P. roquefortii* (Roquefort cheese) and *P. camembertii* (Camembert and Brie cheeses).



12.22 *Penicillium notatum* Colony on Sabouraud Dextrose Agar ♦ The green, granular surface with radial furrows and a white apron are typical of the genus.



12.23 *Penicillium* Conidiophores ♦ *Penicillium* species produce a characteristic brush-shaped conidiophore (penicillus means "brush" in Latin). Metulae (M), phialides (P), and chains of spherical conidia (C) are visible.

Application

Identification of molds and yeasts still largely relies on visual examination of fungal colonies and/or microscopic examination of fungal growth (mycelia or individual cells).

In This Exercise

Today you will examine live yeast and mold cultures, as well as prepared slides of yeasts and molds, as an introduction to their structural diversity.

✓ Materials

Per Student Group

- ☐ Agar slant of *Saccharomyces cerevisiae*
- ☐ Potato dextrose agar or Sabouraud dextrose agar plate culture of *Aspergillus* spp. (with lid taped on)
- ☐ Potato dextrose agar or Sabouraud dextrose agar plate culture of *Penicillium* spp. (with lid taped on)
- ☐ Potato dextrose agar or Sabouraud dextrose agar plate culture of *Rhizopus* spp. (with lid taped on)
- ☐ Gram's iodine stain or methylene blue
- ☐ Dissecting microscope
- ☐ Prepared slides of:
  - *Candida albicans*
  - *Rhizopus* spp. sporangia
  - *Rhizopus* spp. gametangia
  - *Aspergillus* spp. conidiophore
  - *Penicillium* spp. conidiophore

Procedure

Ascomycete Yeasts—*Saccharomyces cerevisiae* and *Candida albicans*

1. Using an inoculating loop and aseptic technique, make a wet mount slide of *Saccharomyces cerevisiae* as illustrated in Figure 3.14, and stain with iodine or methylene blue. Observe under high dry and oil immersion (if the coverslip does not get in the way—observe from the side as you rotate the nosepiece and do not force the oil lens into position if it looks like it will hit the coverslip). Identify vegetative cells and budding cells. Sketch representative cells in the space provided on the data sheet, page 793.
2. Observe prepared slides of *Candida albicans* under high dry and oil. Identify vegetative cells and budding cells. Sketch representative cells in the space provided on the data sheet.

Ascomycete Molds—*Penicillium* and *Aspergillus*

1. Obtain the plate culture of *Penicillium*. **Do not remove the lid from the plate or you will spread spores and contaminate the laboratory.**
   a. Examine the colony morphology and sketch a representative colony in the space provided on the data sheet. Record the ***color*** on both the top (obverse) and bottom (reverse) surfaces. Also record the ***colony texture*** as glabrous (leathery), velvety, yeast-like, cottony, or granular (powdery), and the ***colony topography*** as flat, rugose (with radial grooves), folded, crateriform, verrucose (warty, rough) or cerebriform (brain-like).
   b. Examine the colony with the dissecting microscope. Identify hyphae and conidia. Sketch and label representative examples in the space provided on the data sheet.
2. Observe prepared slides of *Penicillium* conidiophores. Identify the following: hyphae, conidiophores, metulae, phialides, and chains of conidia. Sketch and label representative examples in the space provided on the data sheet.
3. Obtain the plate culture of *Aspergillus*. **Do not remove the lid from the plate or you will spread spores and contaminate the laboratory.**
   a. Examine the colony morphology and sketch a representative colony in the space provided on the data sheet. Record the ***color*** on both the top (obverse) and bottom (reverse) surfaces. Also record the ***colony texture*** as glabrous (leathery), velvety, yeast-like, cottony, or granular (powdery), and the ***colony topography*** as flat, rugose (with radial grooves), folded, crateriform, verrucose (warty, rough), or cerebriform (brain-like).
   b. Examine the colony with the dissecting microscope. Identify hyphae and conidia. Sketch and label representative examples in the space provided on the data sheet.
4. Observe prepared slides of *Aspergillus* conidiophores. Identify hyphae, conidiophores, phialides, and conidia. Sketch and label representative examples in the space provided on the data sheet.

12

Zygomycete—*Rhizopus*

1. Obtain the plate culture of *Rhizopus*. **Do not remove the lid. Uncovering the organism will spread spores and contaminate the laboratory.**
   a. Examine the colony morphology and sketch a representative colony in the space provided on the data sheet. Record the ***color*** on both the top (obverse) and bottom (reverse) surfaces. Also record the ***colony texture*** as glabrous (leathery), velvety, yeast-like, cottony, or granular (powdery), and the ***colony topography*** as flat, rugose (with radial grooves), folded, crateriform, verrucose (warty, rough) or cerebriform (brain-like).
   b. Examine the colony with the dissecting microscope and identify hyphae, rhizoids, sporangiospores, and sporangia. Sketch and label representative structures in the space provided on the data sheet.
2. Examine prepared slides of *Rhizopus* sporangia using medium and high-dry powers. Identify the following: sporangiophores, sporangia, and sporangiospores. Sketch and label representative examples in the space provided on the data sheet.
3. Examine prepared slides of *Rhizopus* gametangia using medium and high-dry powers. Identify the following: progametangia, gametangia, young zygosporangia, mature zygosporangia. You will need to scan the slide at lower magnification to find these, then sketch and label representative examples as viewed with higher magnification in the space provided on the data sheet.

References

Collins, C. H., Patricia M. Lyne, and J. M. Grange. Chap. 51 in *Collins and Lyne's Microbiological Methods*, 7th ed. Oxford, Boston: Butterworth-Heineman, Oxford, 1995.

Fisher, Fran and Norma B. Cook. Chap. 2 in *Fundamentals of Diagnostic Mycology*. Philadelphia: W. B. Saunders, 1998.

Forbes, Betty A., Daniel F. Sahm, and Alice. S. Weissfeld. Chap. 53 in *Bailey & Scott's Diagnostic Microbiology*, 11th ed. St. Louis, MO: Mosby-Year Book, 2002.

Garcia-Hermoso, Dea, Eric Dannaoiu, Olivier Lortholary, and François Dromer. Chap. 119 in *Manual of Clinical Microbiology*, 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock. eds. Washington, DC: ASM Press, 2011.

Kinghorn, J.R. and S.E. Unkles. Chap. 4 in *Biotechnology Handbooks 7 Aspergillus*, J. Smith, ed. New York: Springer, 1994.

Koneman, Elmer W., Stephen D. Allen, William M. Janda, Paul C. Schreckenberger, and Washington C. Winn, Jr. Chap. 19 in *Color Atlas and Textbook of Diagnostic Microbiology*, 5th ed. Philadelphia: J. B. Lippincott, 1997.

Reece, Jane B., Lisa A. Urry, Michael L. Cain, Steven A. Wasserman, Peter V. Minorsky, and Robert B. Jackson. Chap. 31 in *Campbell Biology* 10th ed. Boston: Pearson, 2014.

Tille, Patricia M. Pages 732–734 in *Bailey & Scott's Diagnostic Microbiology*, 13th ed. St. Louis, MO: Mosby, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 12-1

Representative Fungi of Medical and Economic Importance

OBSERVATIONS AND INTERPRETATIONS

1 Sketch your observations of the microscopic structure of the assigned fungi in the appropriate circles below. Take pride in your sketches and label relevant structures. It is more important to draw one or two components in detail than to scribble the entire tangled mass of hyphae.

Ascomycete Yeasts

Saccharomyces cerevisiae
wet mount

(× _____)

Cell dimensions _____

Candida albicans
vegetative cells

(× _____)

Cell dimensions _____

Ascomycete Molds

Penicillium culture

Obverse color _____ Colony texture _____

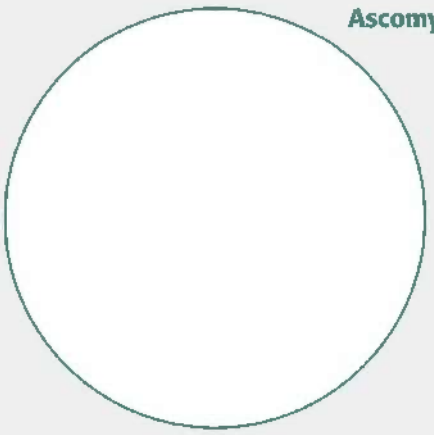
Reverse color _____ Colony topography _____

Penicillium conidiophores and conidia

(× _____)

Conidia dimensions _____

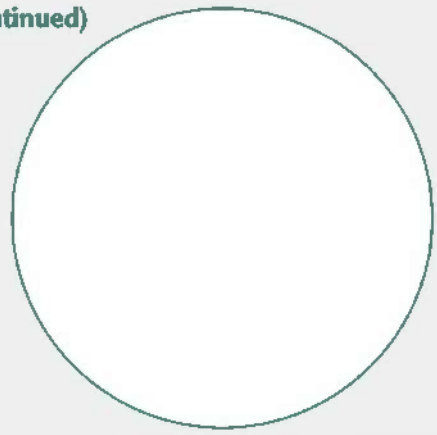
Ascomycete Molds (continued)



Aspergillus colony

Obverse color _____ Colony texture _____

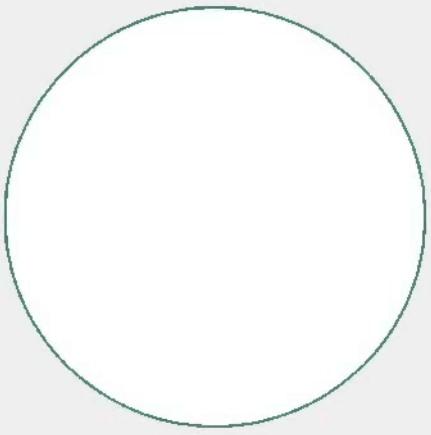
Reverse color _____ Colony topography _____



Aspergillus conidiophores and conidia (× _____)

Conidia dimensions _____

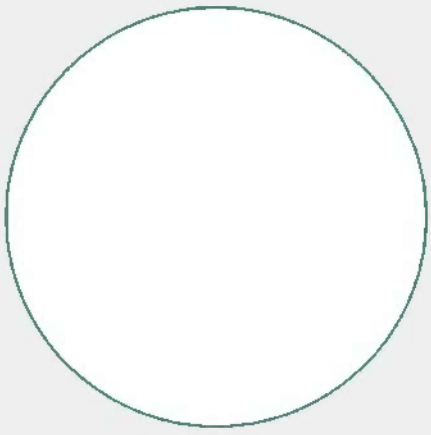
Zygomycota



Rhizopus colony

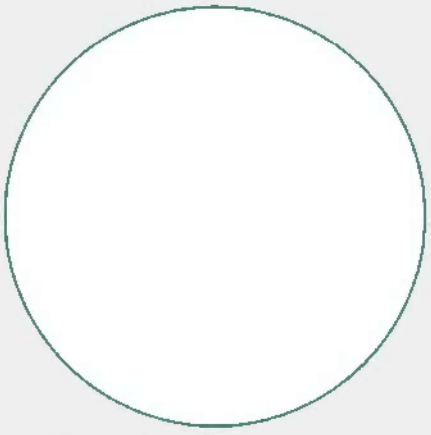
Obverse color _____ Obverse color _____

Reverse color _____ Colony topography _____



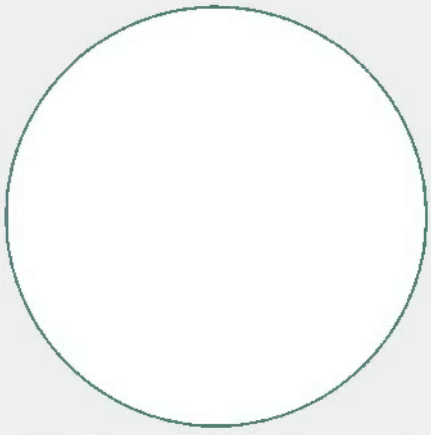
Rhizopus sporangia (× _____)

Sporangium dimensions _____



Rhizopus gametangia (× _____)

Gametangium dimensions _____



Rhizopus zygosporangium (× _____)

Zygosporangium dimensions _____

EXERCISE
12-2
Fungal Slide Culture

Theory

The background material concerning fungi is contained in Exercise 12-1.

Application

Classical fungal taxonomy has relied on the appearance and arrangement of sexual spores, as well as certain features of the hyphae. Because sporangia are so delicate, it is difficult to transfer a portion of mycelium to make a wet mount showing an intact sporangium with its spores. (For an example of what these wet mounts frequently look like, see Figure 12.21 and notice all the scattered, detached spores!) By growing the fungus on a microscope slide, intact sporangia can be observed without disturbing them (Fig. 12.24).

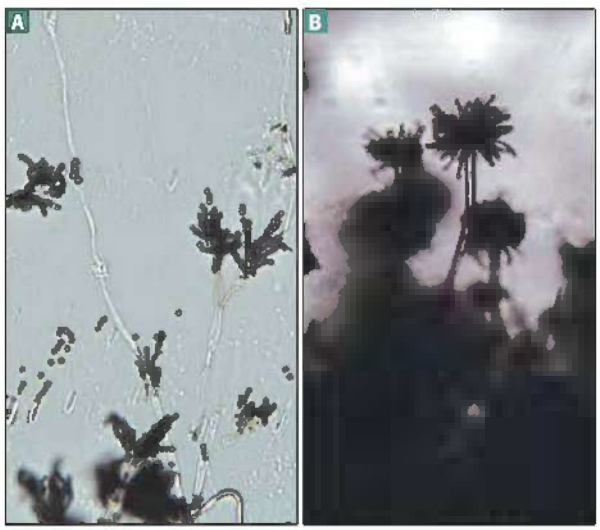
In This Exercise

You will grow a mold in a slide culture and observe its sexual spores.

✓ Materials

Per Class

- ☐ Fume hood or cell culture hood
- ☐ Aerosol disinfectant in hood



12.24 Slide Cultures Illustrating Conidiophores ♦
(A) *Penicillium*, and (B) *Aspergillus*.

Per Student Group

- ☐ Sabouraud agar slant or plate culture of mature (sporing) *Penicillium* spp. (This can be located in the fume or cell culture hood; one culture should serve the entire class.)
- ☐ Sabouraud agar slant or plate culture of mature (sporing) *Aspergillus* spp. (This can be located in the fume or cell culture hood; one culture should serve the entire class.)
- ☐ 5 mL tube of sterile, molten Sabouraud agar (stored in a 50°C water bath until needed)
- ☐ 50 mL beaker with alcohol
- ☐ 50 mL flask of sterile water
- ☐ Bunsen burner
- ☐ Petroleum jelly
- ☐ Sterile toothpicks or wooden applicators
- ☐ Jar of disinfectant large enough to immerse the microscope slides when finished

Per Student

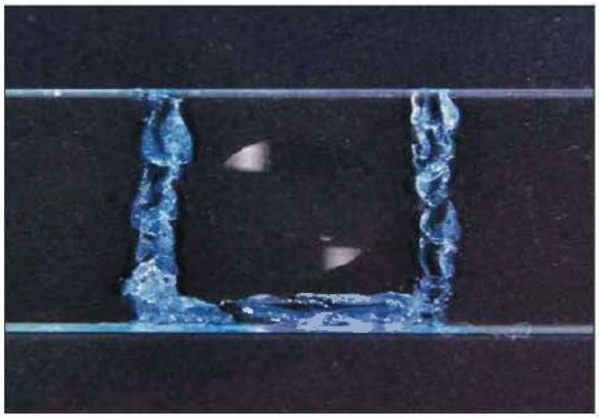
- ☐ Sterile Petri dish
- ☐ Glass rod bent to about 30 degrees in a way it will fit into the Petri dish (a bendable drinking straw will also work)
- ☐ Filter paper cut to fit inside the Petri dish
- ☐ Sterile transfer pipettes
- ☐ Depression microscope slide
- ☐ Cover slips
- ☐ Forceps
- ☐ Inoculating (or dissecting) needle
- ☐ Scalpel
- ☐ Gloves

Procedure

Lab One

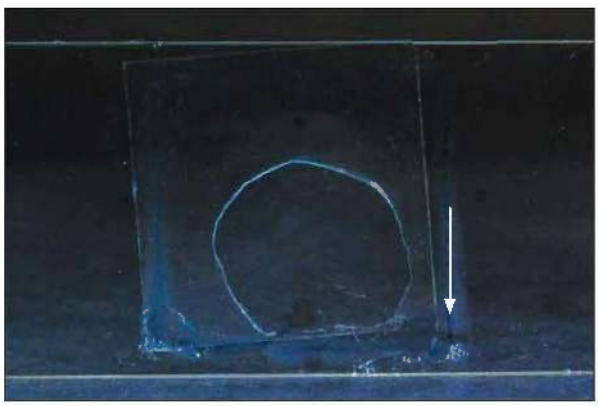
1. Place a folded paper towel on your desk. It should be about the size of a magazine. Put all the working materials on the towel: Petri dish, glass rod, depression slide, cover slip, forceps, inoculating needle, and sterile transfer pipettes.
2. Place the filter paper inside the Petri dish. Set the glass rod on top of the filter paper (Fig. 12.25).

12.25 The Fungal Slide Culture Preliminary Setup ◆ Shown here is the Petri dish, bent glass rod, and moistened filter paper.



12.26 Petroleum Jelly Seal ◆ Use a wooden applicator or toothpick to make a bead of petroleum jelly about the size of the cover slip on three sides of the slide's depression. This is easier to do if the petroleum jelly is slightly warm. In this photo, blue food coloring has been added to the petroleum jelly so it is easier to see.

3. Saturate (but do not flood) the filter paper with sterile distilled water using a sterile transfer pipette. Replace the lid.
4. Using a sterile transfer pipette, rinse the depression slide with alcohol. Tap the excess alcohol off on the paper towel. Then, holding the slide with a forceps pass it *through* the flame and let the alcohol burn off. Do NOT perform this step over the paper towel or the alcohol because a drop of flaming alcohol can start a fire. After it cools, place it on the paper towel with the depression upward.
5. Repeat step 4 with the cover slip. Be sure not to heat the cover slip too much or it will melt or crack.
6. Using the toothpick or wooden applicator, apply a bead of petroleum jelly around the two sides and bottom of the depression on the slide (Fig. 12.26). This process is made easier if the petroleum jelly is slightly warm. Make sure it is applied so its size matches the cover slip's size.
7. Dip the forceps in alcohol, and then pass them through the flame. Let the alcohol burn off and set the forceps aside on the paper towel. Do NOT perform this step over the paper towel or the alcohol, because a drop of flaming alcohol can start a fire.
8. Using a sterile transfer pipette, place a drop of molten Sabouraud agar in the depression of the slide. Immediately place the cover slip over the agar using the sterile forceps. Gently press it down to seal the edges. Allow the agar to solidify (Fig. 12.27).
9. Once the agar has solidified, *gently* slide the cover slip away from the unsealed edge so about two-thirds of the agar is exposed (Fig. 12.27).



12.27 Agar in the Well ◆ Shown here is the solidified agar in the well and covered with the cover slip. The arrow indicates the direction you should slide the cover slip in step 9, that is, away from its unsealed edge.

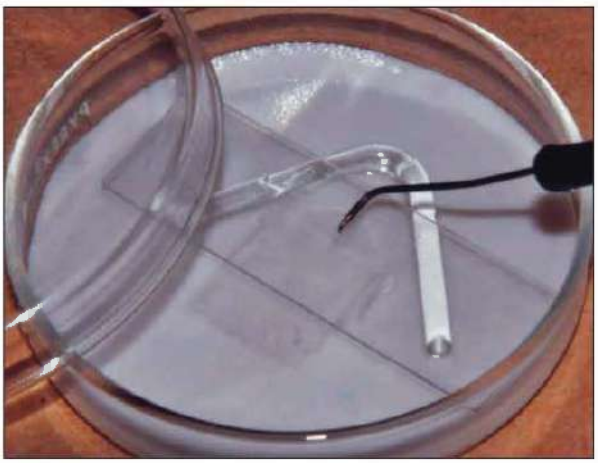
10. Dip the scalpel blade in alcohol, and then pass the blade through the flame. Let the alcohol burn off. Be sure not to catch the scalpel's handle on fire! Also, do NOT perform this step over the paper towel or the alcohol, because a drop of flaming alcohol can start a fire.
11. Gently make a straight cut across the diameter of the agar with the scalpel. Remove the top (uncovered) half.
12. Place the slide on the glass rod inside the Petri dish and replace the lid. Then, take your slide, inoculating needle, forceps, and fungal culture (if located at your desk) to the fume or cell culture hood designated for fungal transfers.

13 Flame the inoculating needle (with alcohol if you are using a dissecting needle—but do not burn the handle!) and touch it to the surface of the fungal growth you are transferring (either *Penicillium* or *Aspergillus*.) Then, gently transfer some fungal growth to the center of the cut edge of agar (Fig. 12.28). Flame the inoculating needle as before inside the hood.

14 With your sterile forceps, gently push the cover slip back over the agar to completely cover it. Do not separate the cover slip from the agar.

15 Replace the cover of the Petri dish and incubate at 25°C for 7 days, or until spores are visible. The plate should be placed right-side up in the incubator.

16 Spray aerosol disinfectant in the air and on the surface of the fume or cell culture hood. Make sure the flame is off.



12.28 Inoculation of the Agar ◆ With the cover slip pulled down out of the way and the agar cut across its diameter, a small amount of fungal mycelium can be transferred to the center of the agar's cut edge. It is difficult to see in the photo, but the tip of the needle is at the agar's edge. After the transfer, the cover slip should be gently pushed back over the entire well and the covered plate incubated at 25°C for 7 days, or until spores are visible.

Lab Two

1 Remove the slide from the Petri dish and examine it under low and high-dry power.

2 Sketch your observations on the data sheet, page 799.

3 Examine another student's preparation of the fungus you did not grow and sketch your observations on the data sheet.

4 Place your slide culture in a jar of disinfectant.

5 Answer the questions on the data sheet.

Reference

Larone, Davise H. *Medically Important Fungi—A Guide to Identification*, 4th ed. Washington, DC: ASM Press, 2002.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 12-2

Fungal Slide Culture

OBSERVATIONS AND INTERPRETATIONS

1 Record your observations of each fungal slide culture in the circles below. Take pride in your sketches and label relevant structures. It is more important to draw one or two components in detail than to scribble the entire tangled mass of hyphae.

Organism _____

Magnification _____

Organism _____

Magnification _____

QUESTIONS

1 *Why is a slide culture used rather than a traditional wet mount for examining molds?*

2 *What is the purpose of the moist filter paper in the slide culture? Why isn't it necessary in bacterial cultures?*

12

Examination of Simple Eukaryotic Pathogens

EXERCISE 12-3

Theory

As discussed in the Introduction to this section, Domain Eukarya has been divided informally (meaning no official taxonomic category has been assigned to them) into four supergroups: Archaeplastida, the SAR clade, Excavata, and Unikonta, with human pathogens being found in the last three. A slightly more detailed description of the supergroups covered in this exercise follows.

- **Supergroup Excavata.** These are unicellular species that usually have a feeding groove on one side of the cell and possess one or more flagella. Many species have a life cycle in which there is an active feeding stage called the **trophozoite** and an inert resting stage called a **cyst**. Subgroups include the diplomonads, parabasalids, and euglenozoans. Each will be discussed with the appropriate organism.
- **Supergroup SAR, subgroup Alveolata.** The unifying feature of this group is the presence of membranous vesicles (alveoli) associated with, but detached from, the plasma membrane. Representative pathogens from the ciliate and apicomplexan subgroups are covered in this exercise.
- **Supergroup Unikonta.** Unikonts form two well-defined subgroups based on molecular evidence: the opisthokonts and the amoebozoans. Their relationship to each other is less clear. Only amoebozoans will be covered in this exercise. (Opisthokonts are discussed in Exercises 12-1 and 12-4.)

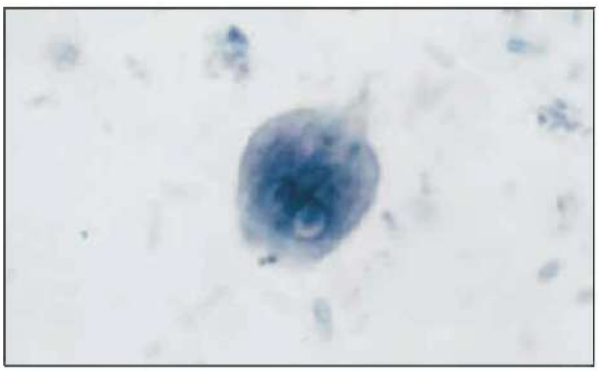
Excavata Pathogens

Giardia lamblia

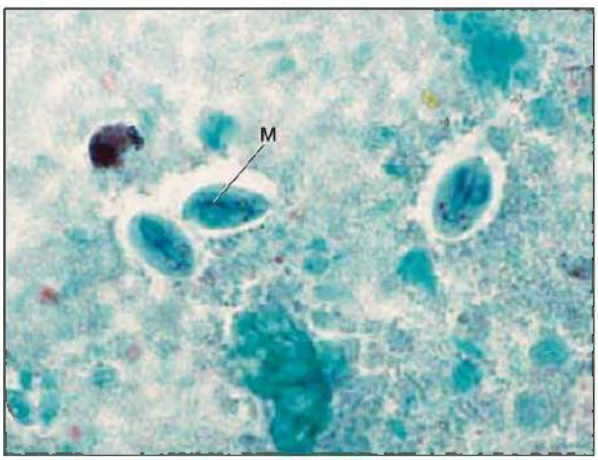
Giardia lamblia (also known as *Giardia intestinalis*) is a diplomonad with worldwide distribution. Diplomonads possess two nuclei of equal size, multiple flagella, and (apparently) degenerated mitochondria called **mitosomes**. Mitosomes lack a functional electron transport chain, thus diplomonads are anaerobes. Their function is unclear.

G. lamblia is the causative agent of giardiasis. It most frequently occupies the duodenum where it feeds on nutrients and secretions. The trophozoite (Fig. 12.29) is heart-shaped with four pairs of flagella and a sucking disk that allows it to resist gut peristalsis. Trophozoites divide longitudinally and produce two daughter trophozoites as a result. Multinucleate cysts (Fig. 12.30) lacking flagella are formed as the organism passes through the colon. Cysts are shed in the feces and may produce infection of a new host upon ingestion. Transmission typically involves fecally contaminated water or food, but direct fecal–oral contact transmission is also possible.

The organism attaches to epithelial cells in the duodenal crypts but does not penetrate to deeper tissues. However, it does damage microvilli and interferes with



12.29 *Giardia lamblia* Trophozoite (Iron Hematoxylin Stain) ◆ Trophozoites have a long, tapering posterior end and range in size from 9 µm to 21 µm by 5 µm to 15 µm. There are two nuclei with small karyosomes. Two median bodies are visible, but the four pairs of flagella are not.



12.30 *Giardia lamblia* Cysts (Trichrome Stain) ◆ Giardia cysts are smaller than trophozoites (8 µm to 12 µm by 7 µm to 10 µm), but the four nuclei with eccentric karyosomes and the median bodies (M) are still visible.

nutrient absorption by the host. Most infections are asymptomatic and clear up on their own. Chronic diarrhea, dehydration, abdominal pain, and other symptoms may occur if the infection produces a population large enough to involve a significant surface area of the small intestine. Diagnosis is made by identifying trophozoites or cysts in stool specimens. Metronidazole or tinidazole are used when treatment is necessary.

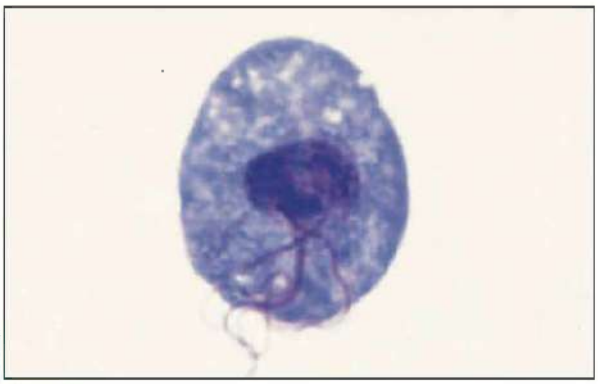
Trichomonas vaginalis

Trichomonas vaginalis (Fig. 12.31) is a parabasalid. Parabasalid motility comes from a group of anterior flagella (*Trichomonas* has 4) and an **undulating membrane**, an extension of the plasma membrane associated with the posterior flagellum. **Hydrogenosomes** are degenerated mitochondria that produce ATP with H_2 gas as the end product.

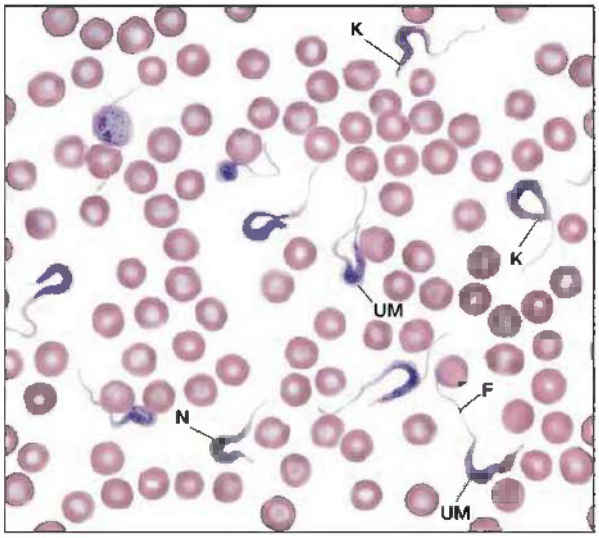
T. vaginalis is the causative agent of trichomoniasis in humans. It causes inflammation of genitourinary mucosal surfaces—typically the vagina, vulva, and cervix in females and the urethra, prostate, and seminal vesicles in males—and infection in females is more common. Most infections are asymptomatic or mild. Some erosion of surface tissues and a discharge may be associated with infection. The degree of infection is affected by host factors, especially the bacterial flora present and the pH of the mucosal surfaces. Transmission typically is by sexual intercourse. Diagnosis is made by microscopic examination of urethral or vaginal discharge and treatment is with metronidazole.

Trypanosoma brucei

Trypanosomes are heterotrophic members of the Euglenozoa, which are characterized by a rod within their flagella in addition to the usual 9 + 2 arrangement of microtubules. *Trypanosoma brucei* (Fig. 12.32) is divided into three subspecies: *T. brucei brucei* (which is nonpathogenic), and *T. brucei gambiense* and *T. brucei rhodesiense*, which cause African trypanosomiasis, also known as African sleeping sickness. The organisms are morphologically very similar but differ in geographic range and disease progress. West African trypanosomiasis (caused by *T. brucei gambiense*) is generally a mild, chronic disease that may last for years before the nervous system is affected, whereas East African trypanosomiasis (caused by *T. brucei rhodesiense*) is more acute and results in death within a year. Modern molecular methods that compare proteins, RNA, and DNA are used to differentiate between them.



12.31 ***Trichomonas vaginalis*** ♦ The trophozoite is the only stage of the *Trichomonas* life cycle and is 7 µm to 23 µm long by 5 µm to 15 µm wide. The four anterior flagella are visible.



12.32 ***Trypanosoma brucei* Trypomastigotes in a Blood Smear** ♦ The central nucleus (N), posterior kinetoplast (K), and undulating membrane (UM) are visible.

Trypanosomes have a complex life cycle. One stage of the life cycle, the **epimastigote**, multiplies in an intermediate host, the tsetse fly (genus *Glossina*). The infective **trypomastigote** stage is then transmitted to the human host through tsetse fly bites. Once introduced, trypomastigotes multiply and produce a chancre at the site of the bite. They enter the lymphatic system and spread through the blood, and ultimately to the heart and brain. Immune response to the pathogen is hampered by the trypanosome's ability to change surface antigens faster than the immune system can produce appropriate antibodies. This antigenic variation also makes development of a vaccine unlikely. Diagnosis is made from clinical symptoms and identification of the trypomastigote in patient specimens (e.g., blood, CSF, and chancre aspirate). An ELISA and an indirect agglutination test also have been developed to detect trypanosome antigens in patient samples.

Progressive symptoms include headache, fever, and anemia, followed by symptoms characteristic of the infected sites. The symptoms of sleeping sickness—sleepiness, emaciation, and unconsciousness—begin when the central nervous system becomes infected. Depending on the

infecting strain, the disease may last for months or years, but the mortality rate is high. Death results from heart failure, meningitis, or severe debility of some other organ(s). As with most diseases, the earlier treatment begins the better the prognosis. A variety of antiprotozoal drugs are used with varying side effects, but those administered later in the disease must cross the blood-brain barrier and are difficult to administer safely.

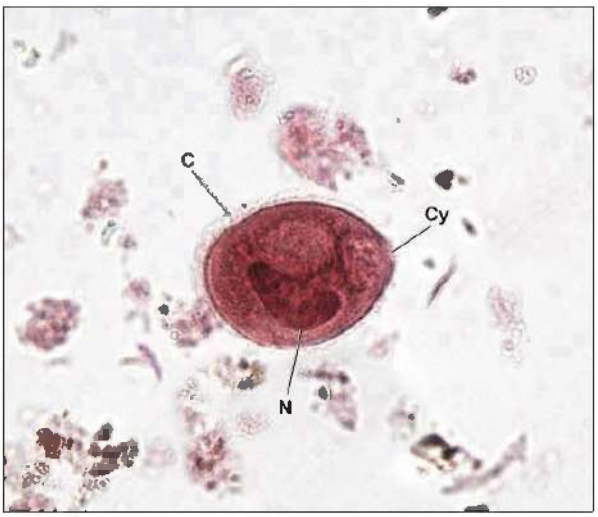
The infective cycle is complete when an infected individual (humans, cattle, and some wild animals are reservoirs) is bitten by a tsetse fly, which ingests the organism during its blood meal. It becomes infective for its lifespan.

Alveolate Pathogens

Balantidium coli

Balantidiasis is found worldwide and is caused by the ciliate pathogen *Balantidium coli*, which exists in two forms: a vegetative trophozoite (Fig. 12.33) and a cyst (Fig. 12.34). Laboratory diagnosis is made by identifying either the cyst or the trophozoite in feces, with the latter being more commonly found. A major risk factor is contact with pigs.

The ciliate trophozoite is highly motile and has a macronucleus and a micronucleus. Cysts in sewage-contaminated water are the infective form and after ingestion become trophozoites in the intestines. Trophozoites reproduce by longitudinal division and live either in the colon's lumen or attach to its mucosa, generally feeding on bacteria. They may cause mucosal ulcerations, but not to the extent produced by *Entamoeba histolytica*. Symptoms of acute infection include bloody and mucoid feces, nausea, vomiting, and abdominal tenderness, among others. Diarrhea alternating with constipation may occur in chronic infections. Most infections probably are asymptomatic. Treatment is with tetracycline or metronidazole.



12.33 *Balantidium coli* Trophozoite ◆ Trophozoites are oval in shape with dimensions of 50 µm to 100 µm long by 40 µm to 70 µm wide. Cilia (C) cover the cell surface. Internally, the macronucleus (N) is prominent; the adjacent micronucleus is not. An anterior cytostome (Cy) is usually visible in most specimens.

12.34 *Balantidium coli* Cyst ◆ Cysts usually are spherical and have a diameter in the range of 50 µm to 75 µm. There is a cyst wall, and the cilia are absent. As in the trophozoite, the macronucleus is prominent, but the micronucleus may not be.

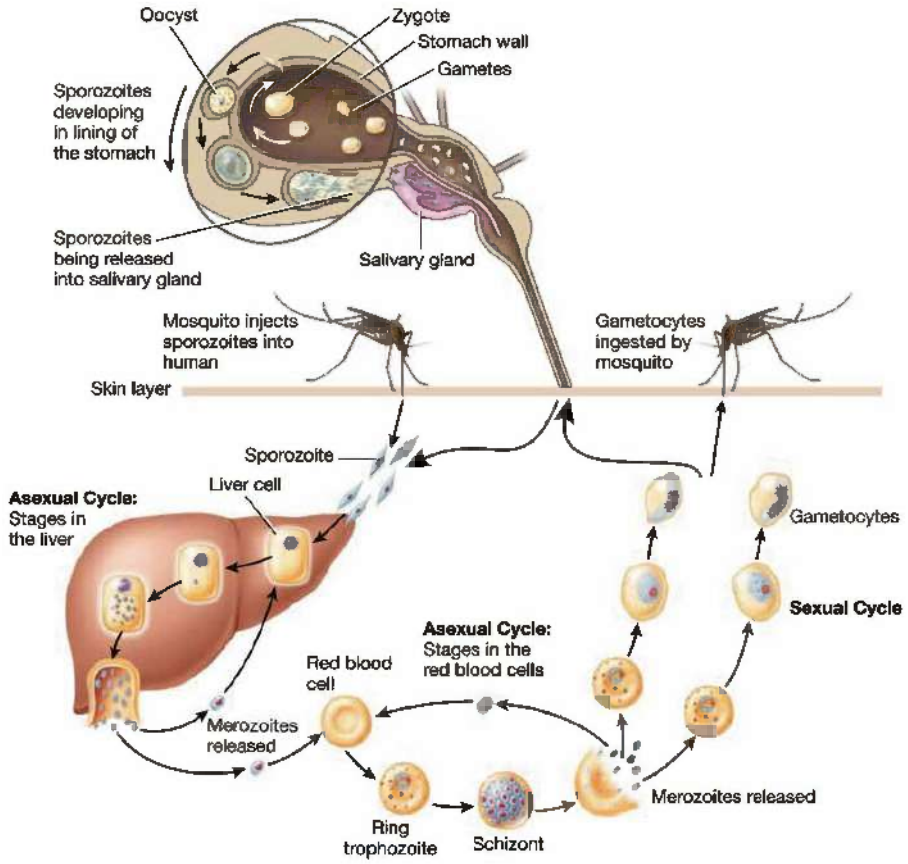
Plasmodium spp.

Plasmodia belong to a group of animal parasites called apicomplexans, which have a complex of organelles at the cell's apex (hence the name) used in penetrating host cells. Plasmodia have a complex life cycle, part of which is in various vertebrate cells while the other part involves an insect. In humans, liver and red blood cells are infected, and the insect vector is the female *Anopheles* mosquito. However, in some parts of the world transmission may also occur via blood transfusion or use of contaminated needles. A generalized life cycle is shown in Fig. 12.35. Representative life cycle stages for the various species are shown in Figures 12.36 to 12.38.

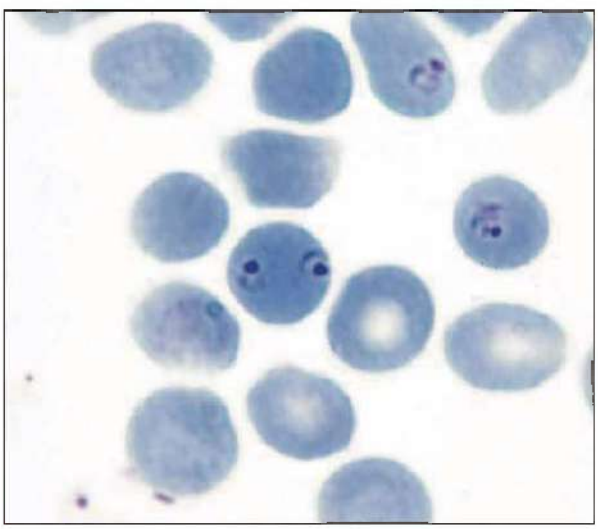
Four species of *Plasmodium* cause different forms of malaria in humans, with the first two accounting for over 90% of the infections:

1. *P. vivax* (benign tertian malaria)
2. *P. falciparum* (malignant tertian malaria)
3. *P. malariae* (quartan malaria)
4. *P. ovale* (ovale malaria)

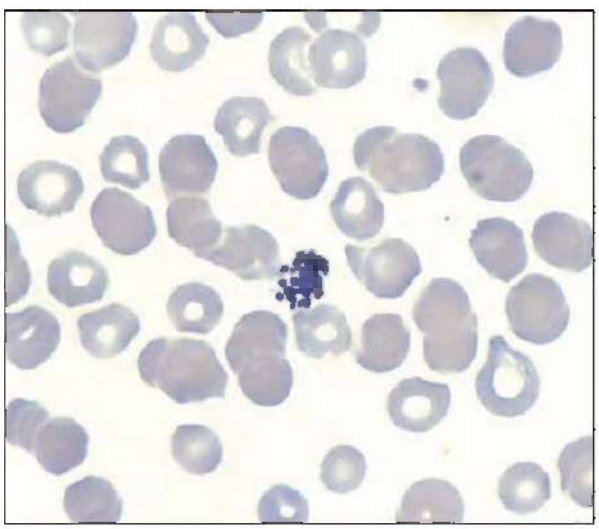
The life cycles are similar for each species, as is the progress of the disease, so the following description is a generic one with only certain specific variations added.



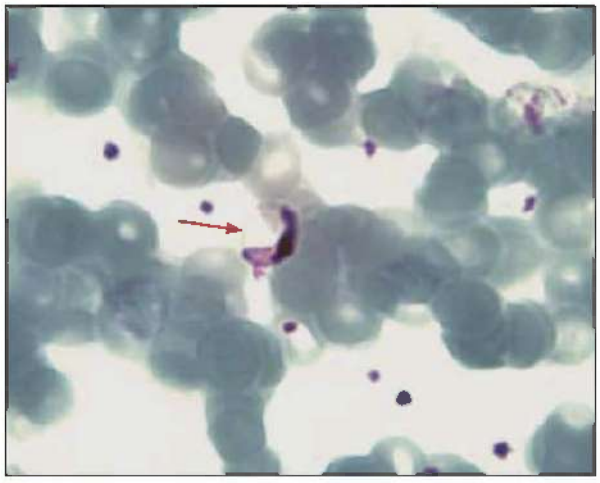
12.35 *Plasmodium* Life Cycle ◆ Please see the text for details.



12.36 *Plasmodium falciparum* Double Infection of a Red Blood Cell ◆ Double infections are commonly seen in *P. falciparum* infections. A single infection is seen at the right. Young trophozoites are said to be in the "ring stage."



12.37 Mature *Plasmodium vivax* Schizont Composed of Approximately 16 Merozoites ◆ More than 12 merozoites distinguish *P. vivax* from *P. malariae* and *P. ovale*, both of which typically have 8, but up to 12 merozoites. *P. falciparum* may have up to 24 merozoites, but they typically are not seen in peripheral blood smears and so are not confused with *P. vivax*.



12.38 *Plasmodium falciparum* Gametocyte in an Erythrocyte ◆ Differentiation between microgametocytes and megagametocytes is difficult in this species. The erythrocyte membrane is visible around the gametocyte (arrow).

The **sporozoite** stage of the pathogen is introduced into a human host during a bite from an infected female *Anopheles* mosquito. Sporozoites then infect liver cells and produce the asexual **merozoite** (trophozoite) stage. Merozoites are released from lysed liver cells, enter the blood, and infect erythrocytes. (Reinfection of the liver occurs at this stage in all except *P. falciparum* infections.) Merozoite reproduction in RBCs goes through a multinucleate **schizont** stage, which undergoes cytokinesis to produce the uninucleate merozoites. (In *P. falciparum* infections, this occurs in visceral capillaries and the schizont is not seen in peripheral blood.) Thus begins a cyclic pattern of reproduction in which more merozoites are produced and released from the red blood cells synchronously.

Merozoite reproductive cycles are tied to the symptoms of malaria. For the first week or so after infection, merozoites are reproducing in the liver and the patient is asymptomatic. Once released from the liver, multiple merozoites infect RBCs, but only one lineage survives and a synchronized periodicity of classic symptoms begins. Erythrocyte rupture and release of merozoites and metabolic wastes lead to chills (lasting an hour or so), fever (lasting a few hours), and sweating (lasting a few hours). These are followed by a return to normal or subnormal body temperature during which merozoites reinfect the red blood cells and the cycle repeats.

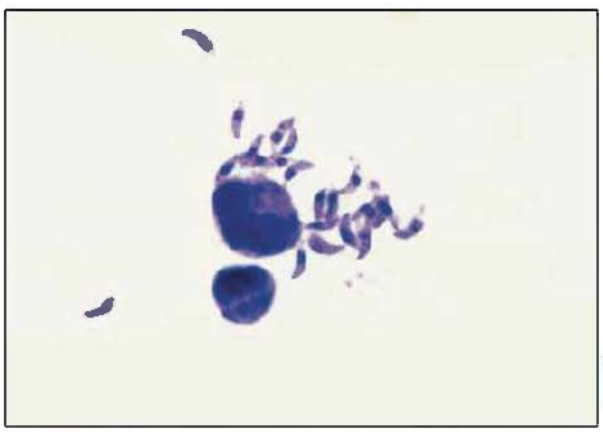
The sexual phase of the life cycle begins when certain merozoites enter erythrocytes and differentiate into male or female **gametocytes**, which are ingested by a female *Anopheles* mosquito during a blood meal. Fertilization occurs in the mosquito and the zygote eventually develops into a cyst within its gut wall. After many divisions, the cyst releases sporozoites, some of which enter the mosquito's salivary glands ready to be transmitted back to the human host.

Most malarial infections are eventually cleared, but not before the patient has developed anemia and has suffered permanent damage to the spleen and liver. The most severe infections involve *P. falciparum*. Erythrocytes infected by *P. falciparum* develop abnormal projections that cause them to adhere to the lining of small blood vessels. This can lead to obstruction of the vessels, thrombosis, or local ischemia, which account for many of the fatal complications of this type of malaria—including liver, kidney, and brain damage. Diagnosis of malaria is made by a combination of appropriate symptoms presented by the patient and a positive identification of the parasite in a blood smear. If speciation is not possible from blood smears, PCR is available.

Treatment is with antimalarial drugs, but a number of factors come into play before a choice is made. These include: *Plasmodium* species involved, parasite load, drug resistance, and patient travel history, among others. Travelers to parts of the world where they may be exposed to malaria are advised to take doxycycline prophylactically before, during, and for four weeks after their trip.

Toxoplasma gondii

Toxoplasma gondii (Fig. 12.39) is an apicomplexan with worldwide distribution and is the causative agent of toxoplasmosis. It is an obligate intracellular parasite. Its life cycle has sexual and asexual phases, with the sexual phase only occurring in the lining of cat intestines, where thick-walled, resistant **oocysts** are produced and shed in the feces. Each mature oocyst contains eight sporozoites and if ingested by a cat, the sexual cycle may be repeated as the sporozoites produce **gametocytes**, which in turn produce gametes, undergo fertilization and produce another oocyst generation.



12.39 *Toxoplasma gondii* trophozoites ◆ Notice the bow-shaped cells and the prominent nuclei.

Ingestion of an oocyst by an animal or human host begins the asexual cycle. Oocyst germination occurs in the duodenum and the released sporozoites infect the epithelial cells. After division, rapidly dividing, motile **tachyzoites** (trophozoites) are released into the blood and spread the infection to lymph nodes and other parts of the reticuloendothelial system (e.g., monocytes). As the infection progresses, tachyzoites differentiate into slowly dividing **bradyzoites**, which in turn form **tissue cysts** that may reside in the host's skeletal muscle, liver, heart, or brain for the rest of its life. Tissue cysts ingested by a cat eating an infected animal develop into gametocytes in the cat's intestines. Gametes are formed, fertilization produces an oocyst, and the life cycle is completed.

Ingestion of oocysts or tissue cysts and transplacental transmission to a fetus are the main routes of human infection. Acute infection of immunocompetent individuals results in mild cold or flu-like symptoms, swollen cervical lymph nodes, or there may be no symptoms at all. Once tissue cysts are formed, spreading of the pathogen usually stops and any symptoms disappear. The more serious form of the disease involves infection of a fetus across the placenta (especially in the first or second trimester) from an infected mother. This type of infection may result in stillbirth, or liver damage and brain damage. Infection of immunocompromised patients or rupturing of an existing tissue cyst typically leads to CNS infection and subsequent alteration of neurological functioning.

Unlike many of the other pathogens in this exercise, diagnosis is not routinely made microscopically because *Toxoplasma* is found mostly within cells that are not easily sampled (as opposed to blood pathogens, for example). Instead, diagnosis usually is done by testing for *Toxoplasma* antibodies. Various drugs are available for treatment, when necessary, such as for infected pregnant women, newborns, and immunocompromised patients.

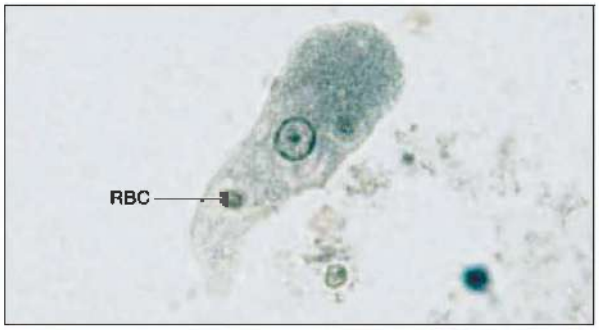
Unikont Pathogen

The only simple unikont pathogens are in the subgroup Amoebozoa. The ability to extend their cytoplasm into rounded pseudopods (in many directions at once!) is a defining feature of these organisms. Pseudopods provide a means of locomotion and feeding by engulfing prey. They, too, have trophozoite and cyst stages.

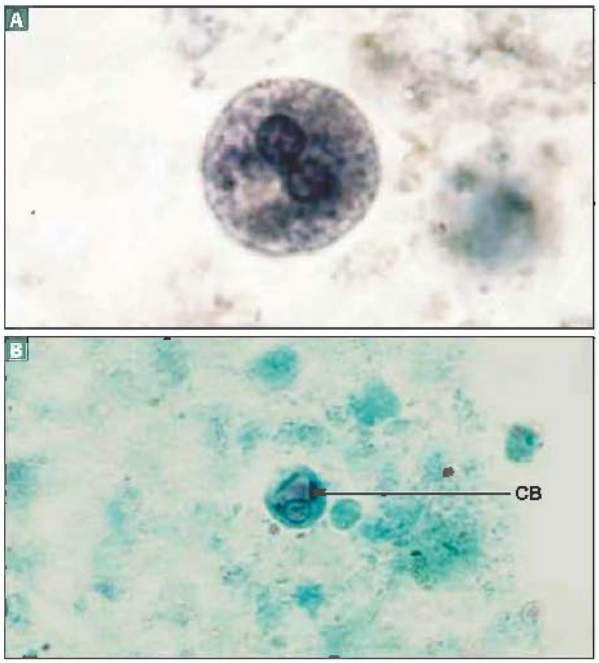
Entamoeba histolytica

Entamoeba histolytica is the causative agent of amebic dysentery (amebiasis), a disease most common in areas with poor sanitation worldwide, but especially regions with warmer climates. Identification is made by finding either trophozoites (Fig. 12.40) or cysts (Fig. 12.41) in a stool sample, though serological tests and PCR have been shown to be more sensitive and specific in differentiating *E. histolytica* from *E. dispar*, a nonpathogenic species morphologically indistinguishable from *E. histolytica*. However, you will be examining slide preparations in this lab, so the microscopic diagnostic features of trophozoites and cysts are used and described in the figure captions.

Infection occurs when a human host ingests cysts, either through direct fecal–oral contact or contaminated food or water. It is the ability of cysts to withstand the



12.40 *Entamoeba histolytica* Trophozoite (Iron Hematoxylin Stain) ◆ Trophozoites range in size from 12 µm to 60 µm. Notice the small, central karyosome, the beaded chromatin at the margin of the nucleus, the ingested red blood cells (RBC), and the finely granular cytoplasm. Compare with an *Entamoeba coli* trophozoite in Figure 12.42.

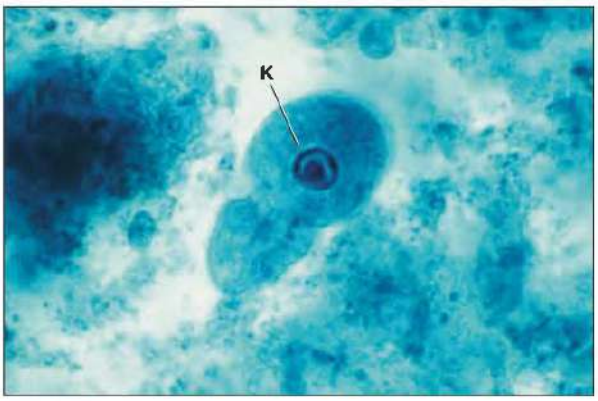


12.41 *Entamoeba histolytica* Cysts ◆ (**A**) Cysts are spherical with a diameter of 10 µm to 20 µm. Two of the four nuclei are visible; other nuclear characteristics are as in the trophozoite. Compare with an *Entamoeba coli* cyst in Figure 12.43 (×1320, iron hematoxylin stain). (**B**) *E. histolytica* cyst (×1200, trichrome stain) with cytoplasmic chromatoidal bars (CB). These are found in approximately 10% of the cysts, have blunt ends, and are composed of ribonucleoprotein.

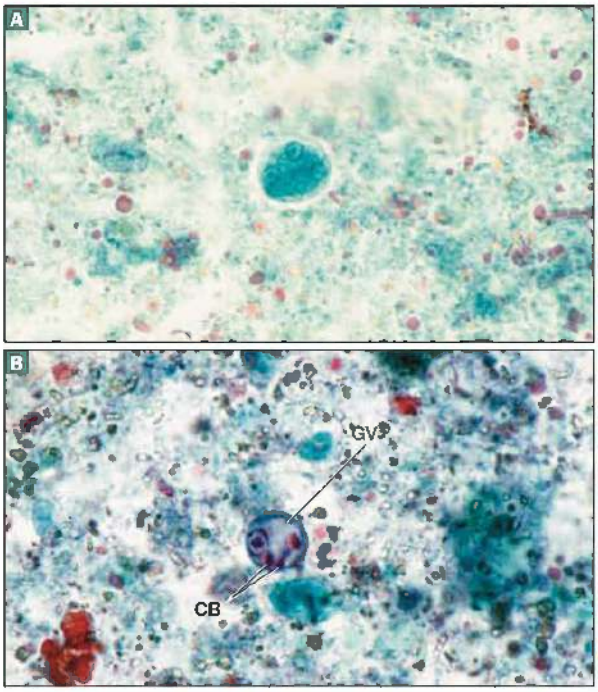
stomach's acidic environment that makes them the infective stage; they are able to deliver trophozoites safely to the less harsh environment of the small intestine. Excystation in the small intestine results in release of one tetranucleate trophozoite in which each nucleus goes through one mitotic division (producing eight nuclei) and the cell does three rounds of cytokinesis to produce eight small uninucleate trophozoites. Trophozoites live in the colon and feed on bacteria. As a consequence of relative dehydration (the colon's primary job is to reabsorb water from the undigested, unabsorbed food in it), trophozoites convert into cysts that are passed in the feces. Mature cysts have four nuclei. They are hardy and able to survive several days outside the host, which makes them problematic as contaminants of water and food. If ingested, the life cycle is completed.

Most infections (~90%) are asymptomatic, because the trophozoites form colonies in the mucus lining the large intestine and feed there. Invasive amebiasis occurs when trophozoites penetrate the mucus layer, attach to a specific epithelial receptor, and cause ulceration of the mucosa leading to acute amebic dysentery, fulminating colitis, or appendicitis. In the most severe cases, trophozoites enter the blood and infect other organs, especially the liver, lungs, or brain. Infections can be treated with tissue amebicides (e.g., metronidazole) that target trophozoites and/or luminal amebicides that target cysts.

Another member of the genus *Entamoeba* deserves mention here. *Entamoeba coli* is a fairly common, nonpathogenic intestinal commensal that must be differentiated from *E. histolytica* in stool samples. Its characteristic features are given in the captions to Figures 12-42 and 12-43.



12.42 ***Entamoeba coli* Trophozoite (Trichrome Stain)** ◆ Trophozoites range in size from 15 µm to 50 µm. Notice the relatively large and eccentrically positioned karyosome (K), the unclumped chromatin at the periphery of the nucleus, and the vacuolated cytoplasm lacking ingested red blood cells. The usually nonpathogenic *E. coli* must be distinguished from the potentially pathogenic *E. histolytica*, so compare with Figure 12.40.



12.43 ***Entamoeba coli* Cysts** ◆ Cysts typically are spherical and are between 10 µm and 35 µm in diameter. They contain 8, or sometimes 16 nuclei. This makes differentiation from *E. histolytica* cysts simpler, because they never have more than 4 nuclei. **(A)** Five nuclei are visible in this specimen (×1000, trichrome stain). **(B)** Chromatoidal bars (CB) and a large glycogen vacuole (GV) characteristic of immature cysts are visible in this specimen (×1000, trichrome stain).

Application

Medical microbiologists rely heavily on microscopic examination of patient samples (e.g., blood and feces) to identify simple eukaryotic pathogens.

In This Exercise

Today you will examine prepared slides of simple pathogenic eukaryotes as an introduction to their cellular diversity and relate what you see to their life cycles and ability to cause disease.

✓ Materials

Per Student Group

☐ Prepared slides of:

- *Giardia lamblia* trophozoites and cysts
- *Trichomonas vaginalis* trophozoites
- *Trypanosoma* spp.
- *Balantidium coli* trophozoites and cysts
- *Plasmodium* spp.

12

- *Toxoplasma gondii* trophozoites
- *Entamoeba coli* trophozoite and cyst
- *Entamoeba histolytica* trophozoites and cysts

Procedure

1. Obtain prepared slides of the simple eukaryotic pathogens and observe them under appropriate magnification. You should observe the assigned structures on each organism. (Many of these slides are made from patient samples, so there will be a lot of other material on the slide besides the desired organism. You must search carefully and with patience.)
2. Sketch and label the assigned components on the data sheet, page 809.

Giardia lamblia

- Trophozoite

  Identify oval shape, flagella (four pairs), nuclei (two), and median bodies (two)

- Cyst

  Identify multiple nuclei (four) and median bodies (four)

Trichomonas vaginalis trophozoite

Identify nucleus and flagella (four)

Trypanosoma spp.

Identify nucleus, flagellum, undulating membrane, and kinetoplast

Balantidium coli

- Trophozoite

  Identify elongated shape, cilia, macronucleus, and micronucleus (maybe)

- Cyst

Plasmodium spp.

Identify ring stage, mature trophozoite, schizont, male gametocyte, and female gametocyte

Toxoplasma gondii

Identify trophozoite (bow-shaped tachyzoites) with prominent spherical nucleus

Entamoeba histolytica

- Trophozoite

  Identify pseudopods; beaded nucleus with small, central karyosome; and ingested erythrocytes

- Cyst

  Identify multiple nuclei (up to four) with karyosomes and chromatin as in the trophozoite, and cytoplasmic chromatoidal bars (maybe)

Entamoeba coli

- Trophozoite

  Identify the same as *E. histolytica* except with eccentric karyosome and unclumped chromatin

- Cyst

  Identify up to 8 nuclei (more than 4 is enough to distinguish it from *E. histolytica*) that are the same as in the trophozoite, and cytoplasmic chromatoidal bars (maybe)

References

Ash, Lawrence R. and Thomas C. Orihel. Chap. 14 in *Parasites: A Guide to Laboratory Procedures and Identification.* Chicago: American Society for Clinical Pathology, 1991.

Bruckner, David A. and Jaime A. Labarca. Chap. 134 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Garcia, Lynne. Chaps. 2, 3, 4, 6, 7, and 9 in *Diagnostic Medical Parasitology,* 5th ed. Washington, DC: ASM Press. 2007.

Koneman, Elmer W., Stephen D. Allen, William M. Janda, Paul C. Schreckenberger, and Washington C. Winn, Jr. Chap. 20 in *Color Atlas and Textbook of Diagnostic Microbiology,* 5th ed. Philadelphia: J.B. Lippincott Company. 1997.

Lee, John J., Seymour H. Hutner, and Eugene C. Bovee. *Illustrated Guide to the Protozoa.* Lawrence, KS: Society of Protozoologists. 1985.

Markell, Edward K., Marietta Voge, and David T. John. *Medical Parasitology,* 7th ed. Philadelphia: W.B. Saunders Company, 1992.

McAuley, James B, Jeffrey L. Jones, and Kamaljit Singh. Chap. 135 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Reece, Jane B., Lisa A. Urry, Michael L. Cain, Steven A. Wasserman, Peter V. Minorsky, and Robert B. Jackson. Chap. 28 in *Campbell Biology* 10th ed. Boston: Pearson, 2014.

Rogers, William O. Chap. 133 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Tille, Patricia M. Chaps. 48, 49, and 50 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis, MO: Mosby, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 12-3

Examination of Simple Eukaryotic Pathogens

OBSERVATIONS AND INTERPRETATIONS

1 Carefully sketch your observations of one or two examples of the assigned organisms in the appropriate circles below.

Supergroup Excavata

Giardia lamblia
trophozoite

(× _____)

Dimensions _____

Giardia lamblia
cyst

(× _____)

Dimensions _____

Trichomonas vaginalis
trophozoite

(× _____)

Dimensions _____

Trypanosoma spp.

(× _____)

Dimensions _____

Balantidium coli
trophozoite

(× _____)

Dimensions _____

Balantidium coli
cyst

(× _____)

Dimensions _____

Plasmodium spp.
ring and mature trophozoites

(× _____)

Dimensions _____

Plasmodium spp.
schizont

(× _____)

Dimensions _____

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 12-3

(continued)

Plasmodium spp.
gametocytes

(× _____)

Dimensions _____

Toxoplasma gondii
trophozite

(× _____)

Dimensions _____

Supergroup Unikonta

Entamoeba histolytica
trophozoite

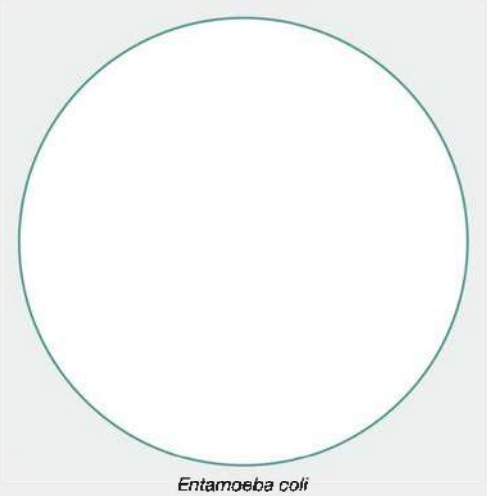
(× _____)

Dimensions _____

Entamoeba histolytica
cyst

(× _____)

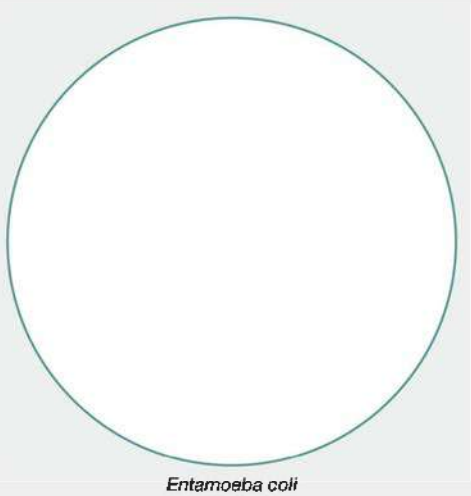
Dimensions _____



Entamoeba coli
trophozoite

(× _____)

Dimensions _____



Entamoeba coli
cyst

(× _____)

Dimensions _____

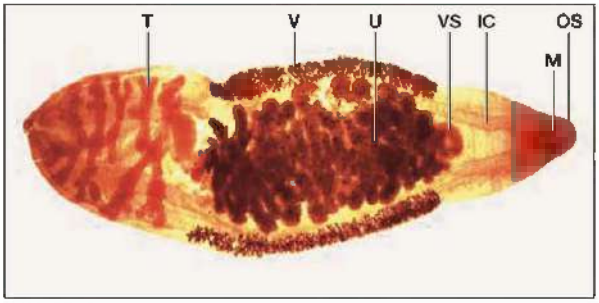
12

EXERCISE
12-4

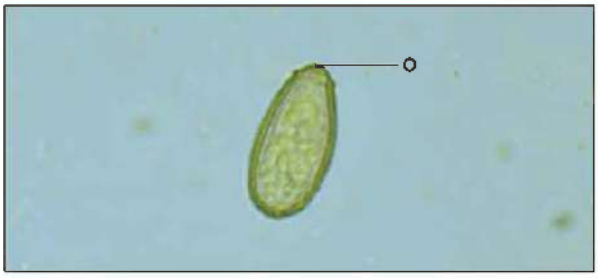
Parasitic Helminths

Theory

A study of helminths is appropriate to the microbiology lab, because clinical specimens may contain microscopic evidence of helminth infection. The three major groups of parasitic worms encountered in lab situations are trematodes (flukes), cestodes (tapeworms), and nematodes (roundworms). Life cycles of the parasitic worms are often complex, sometimes involving several hosts, and are beyond the scope of this book. Emphasis here is on a brief background and clinically important diagnostic features of selected worms.



12.44 ***Clonorchis sinensis* Adult** ♦ Adults range in size from 1 cm to 2.5 cm. Visible in this specimen are the oral sucker (OS), mouth (M), intestinal ceca (IC), ventral sucker (VS), uterus (U), vitellaria (V), and testis (T).



12.45 ***Clonorchis sinensis* Egg in a Fecal Sample (D'Antoni's Iodine Stain)** ♦ The eggs are thick-shelled and between 27 µm and 35 µm long. There is a distinctive operculum (O) (positioned to give the appearance of shoulders) and often a knob at the abopercular end (not visible in this specimen).

Trematode Parasites Found in Clinical Specimens

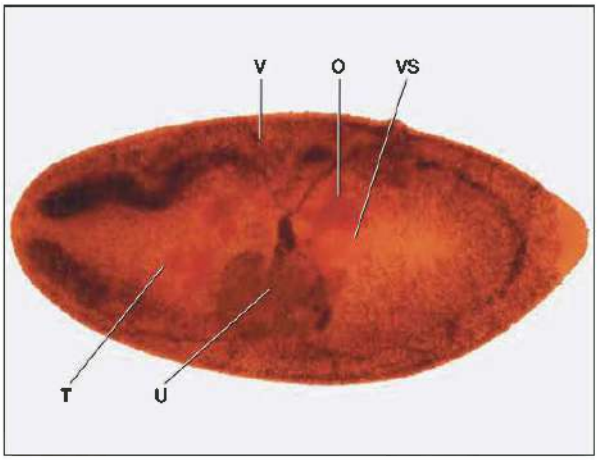
Trematode worms belong to the phylum Platyhelminthes, which literally means "flat worm" and is their common name as well as a description of their shape. Flatworms lack a body cavity, true organs, and (usually) have only one opening to their digestive tract. The class Trematoda is composed largely of parasitic species with their life cycles involving two or more hosts (one definitive host—a vertebrate—and one or more intermediate hosts—frequently a mollusc such as a snail) and multiple developmental stages. The larval stages reproduce asexually, but the adult worms, which live in the definitive host, are generally hermaphroditic and represent the sexual stage of the life cycle.

Clonorchis sinensis

Clonorchis sinensis is the Chinese liver fluke (Fig. 12.44), which causes clonorchiasis, a liver disease. It is a common parasite of people living in China, Japan, Korea, Vietnam, and Taiwan, and is becoming more common in the United States with the influx of Southeast Asian immigrants. Infection typically occurs when undercooked infected fish is ingested. The adults migrate to the liver bile ducts and begin laying eggs in approximately one month. Most infections are asymptomatic, but patients may have a fever, abdominal pain, and jaundice. Damage to the bile duct epithelium, liver, and gallbladder is related to the number of worms and the duration of infection. Diagnosis is made by identifying the characteristic eggs in feces (Fig. 12.45), and treatment is with praziquantel. Preventative measures include improved sanitation and thorough cooking of fish and freshwater plants.

Paragonimus westermani

Paragonimus westermani (Fig. 12.46) is a lung fluke and one of several species to cause paragonimiasis, a disease mostly found in Asia, Africa, and South America. *P. westermani* is primarily a parasite of carnivores, but humans (omnivores) may get infected when eating undercooked crabs or crayfish infected with the cysts. Ingested juveniles excyst in the duodenum, penetrate the intestinal wall and diaphragm, and find their way to the lungs where the adults mature. Eggs (Fig. 12.47) are released into the bronchioles in approximately two to three months and are diagnostic of infection. They may be passed in sputum or feces (as a result of coughing and swallowing) and find their way to the first intermediate host, which is a freshwater snail. Development continues in a second intermediate host, crabs or crayfish, which completes the life cycle.



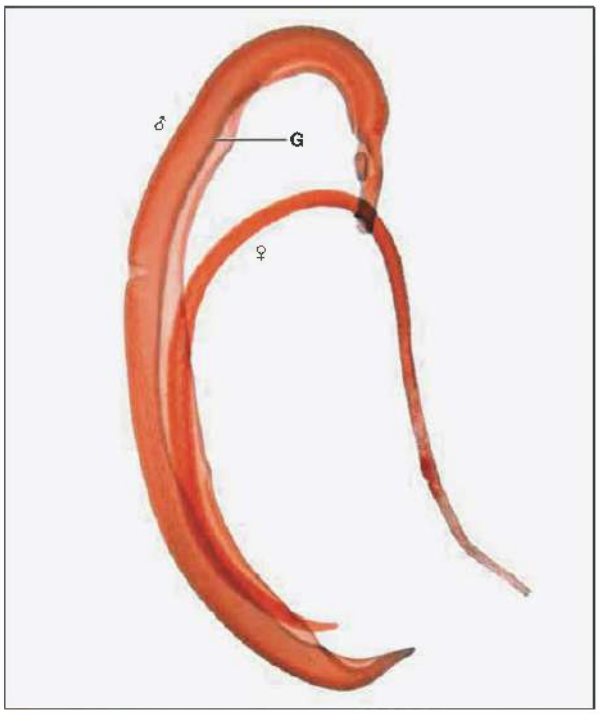
12.46 *Paragonimus westermani* Adult ◆ Adults are up to 1.2 cm long, 0.6 cm wide, and 0.5 cm thick. Visible in this specimen are the ventral sucker (VS), ovary (O), uterus (U), testis (T), and vitellaria (V).

12.47 *Paragonimus westermani* Egg in a Fecal Sample (D'Antoni's Iodine Stain) ◆ *Paragonimus westermani* eggs are ovoid and range in size from 80 µm to 120 µm long by 45 µm to 70 µm wide. They have an operculum (O) and the shell is especially thick at the abopercular end (arrow). They are unembryonated when seen in feces.

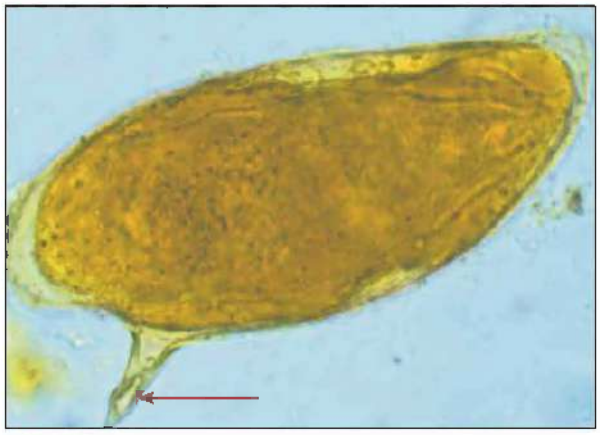
Consequences of lung infection are a local inflammatory response followed by possible ulceration. Symptoms include cough with discolored or bloody sputum and difficulty breathing. These cases are rarely fatal, but may last a couple of decades. If the parasite load is small, the patient may experience no symptoms, though migration of juveniles through the body may cause pain. Occasionally, the wandering juveniles end up in other tissues, such as the brain or spinal cord, which can cause paralysis or death. Diagnosis is made by finding eggs in feces or sputum or by serological testing for *P. westermani* antibodies in the patient. Treatment is with praziquantel, but infection can be avoided by proper cooking of crabs and crayfish and better sanitation.

Schistosoma mansoni

Schistosoma mansoni (Fig. 12.48) is one of four blood fluke species that infect humans. All blood flukes have a more rounded body than typical flatworms, an oral and ventral sucker, and separate sexes. *S. mansoni* is found in Brazil, some Caribbean islands, Africa, and parts of the Middle East. Infection occurs via contact with fecally contaminated water containing juveniles of the species released from the snail intermediate host. The juveniles penetrate the skin, enter circulation, and continue development in the venules of the large intestine. Eggs (Fig. 12.49) from the adults penetrate the intestinal wall and are passed out with the feces. Presence of eggs in the feces indicates infection. Some patients are asymptomatic, whereas others have bloody diarrhea, abdominal pain, and lethargy. Treatment is with praziquantel, but prevention is problematic because infection occurs through the skin, which requires better water quality for bathing and laundry.



12.48 *Schistosoma mansoni* Adults ◆ The male is shorter (~1.5 cm) and wider than the female and has a gynecophoral groove (G) in which the longer (~2 cm), more slender female resides during mating.



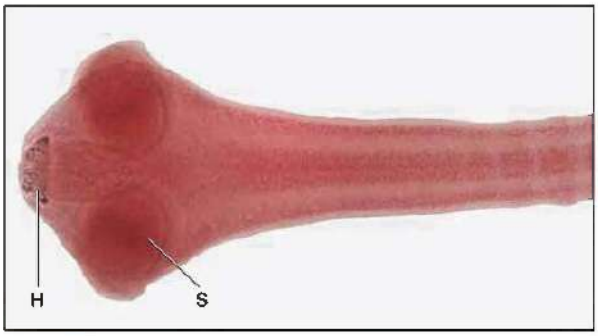
12.49 *Schistosoma mansoni* Egg in a Fecal Sample (D'Antoni's Iodine Stain) ◆ *Schistosoma mansoni* eggs are large (114 µm to 175 µm long by 45 µm to 70 µm wide) and contain a larva called a miracidium. They are thin-shelled, lack an operculum, and have a distinctive lateral spine (arrow).

Cestode Parasites Found in Clinical Specimens

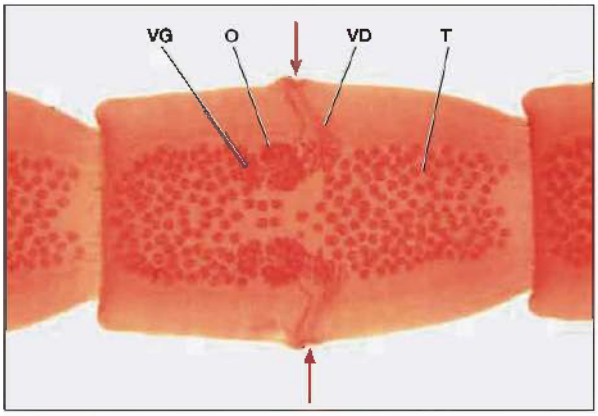
Cestodes, commonly called tapeworms, are a class of flatworms composed exclusively of vertebrate intestinal parasites. The adult body is divided into segments called **proglottids**, each of which is devoted exclusively to sexual reproduction and carries both egg and sperm producing structures. The **scolex** (head) is modified with suckers, hooks, or both, for attachment to the intestinal wall where they may reside for years. The head may also have an evertable **rostellum**. No mouth or digestive tract is necessary, because they absorb nutrients from the intestinal contents through their body surface.

Dipylidium caninum

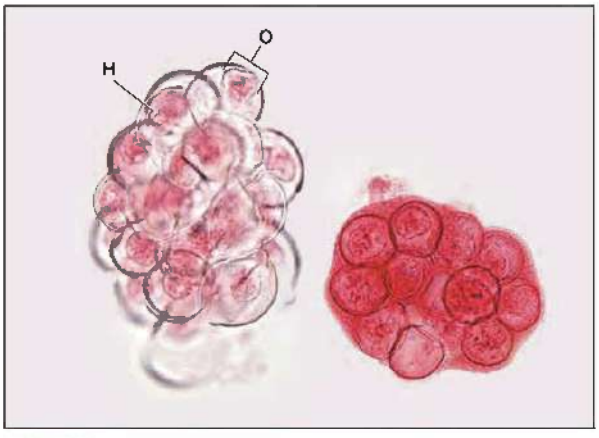
Dipylidium caninum (Fig. 12.50) is a common parasite of dogs and cats worldwide. The adult worms reside in dog or cat intestines and release proglottids (Fig. 12.51) containing egg packets that migrate out of the anus. When these dry, they look like rice grains. Larval fleas (the intermediate host) may eat the eggs and become infected. If the dog, cat, or human—frequently a child due to proximity to the cat or dog pet—ingests one of these fleas, the life cycle is completed in the new host. Infection may be asymptomatic or produce mild abdominal discomfort, appetite and weight loss, perianal itching, and indigestion. Diagnosis is made by identifying the egg packets (Fig. 12.52). Treatment is usually not necessary because infection is frequently self-limiting and asymptomatic, but praziquantel may be prescribed for humans and their pets.



12.50 *Dipylidium caninum* Scolex ◆ Adult worms may reach a length of 50 cm with a width of 3 mm. The scolex has four suckers (S) and a retractable rostellum with rows of hooks (H).



12.51 *Dipylidium caninum* Proglottids ◆ Visible are the testes (T), vasa deferentia (VD), ovaries (O), and vitelline glands (VG). The reproductive openings (arrows) on each side of the proglottid give this worm its common name—the "double-pored tapeworm."

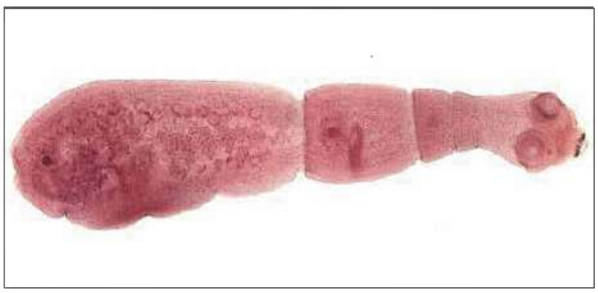


12.52 *Dipylidium caninum* Egg Packet ◆ Each *Dipylidium caninum* egg packet is composed of 5 to 15 eggs, each with an oncosphere (O). The oncosphere has six hooklets (H).

Echinococcus granulosus

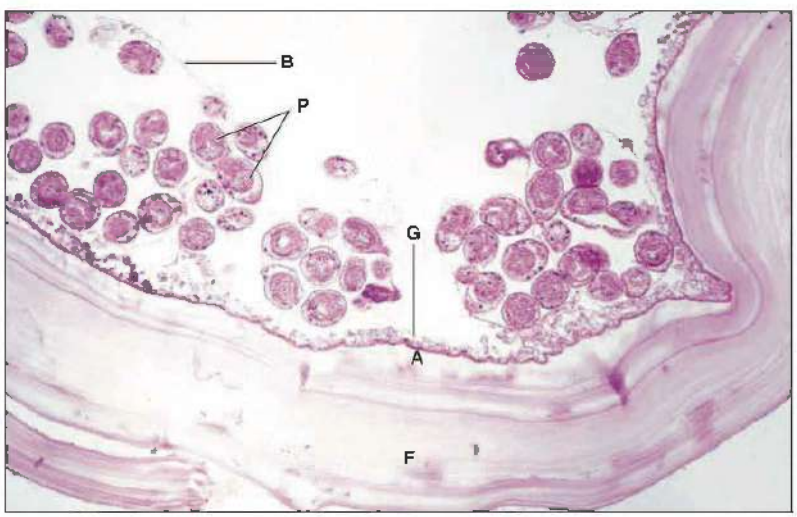
The definitive host of *Echinococcus granulosus* (Fig. 12.53) is a carnivore, but the life cycle requires an intermediate host, usually an herbivorous mammal. If an herbivore eats the eggs passed in the carnivore's feces, they develop into thick-walled **hydatid cysts** (Figs. 12.54 and 12.55) containing **protoscolices** (juvenile worms) in its lungs, liver, or other organ. When a carnivore eats an infected intermediate host, the protoscolices develop into adult worms in its intestines and the life cycle is completed.

Humans involved in raising domesticated herbivores (e.g., sheep with their associated dogs) are most susceptible to infection, but are dead-end hosts as the parasite cannot reproduce and humans are not likely to be eaten. Instead, infected humans develop hydatid disease, in which hydatid cysts form in the lung, liver, or other organ, a process that may take many years. Hydatid disease is found in countries around the Mediterranean Sea, South America, and Africa, but only sporadically in the United States. Symptoms depend on the location and size of the hydatid cyst, which interferes with normal organ function. Due to sensitization by the parasite's antigens, release of fluid from the cyst can result in potentially fatal anaphylactic shock of the host. Diagnosis is made by detection of the cyst by CT scan, ultrasound, or X-ray. Treatment can involve antiparasitic medications, aspiration and sterilization of the cyst's contents, or surgical removal.



12.53 *Echinococcus granulosus* Adult ◆ Adult worms are small, reaching a length of about 0.5 cm. There is a scolex with a ring of hooks and suckers, a neck, and a posterior proglottid that contains up to 1,500 eggs.

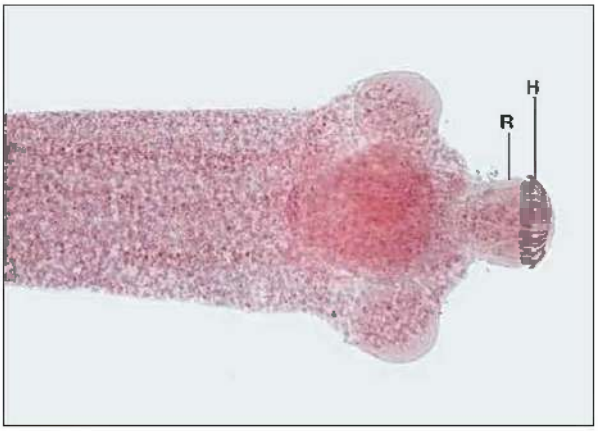
12.55 *Echinococcus granulosus* Protoscolex (l.s.) ◆ The protoscolex contains an invaginated scolex with hooks (H). Upon ingestion by the host, the protoscolex evaginates and produces an infectious scolex that attaches to the intestinal wall, matures, and produces eggs.



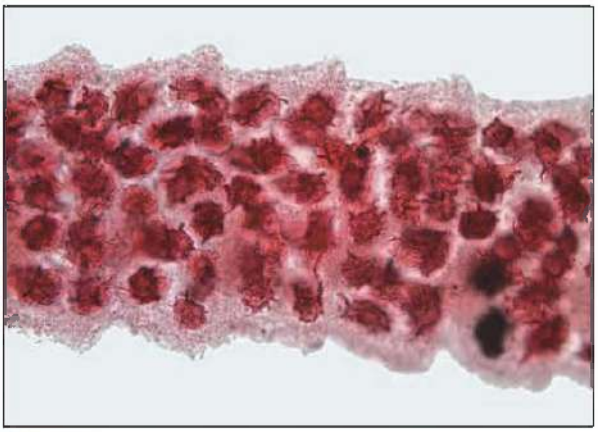
12.54 *Echinococcus granulosus* Cyst in Lung Tissue (sec.) ◆ The cyst wall consists of a host tissue fibrous layer (F), an acellular layer (A), and a germinal epithelium (G) that gives rise to stalked brood capsules (B). Brood capsules produce many *E. granulosus* protoscolices (P).

Hymenolepis (Vampirolepis) nana

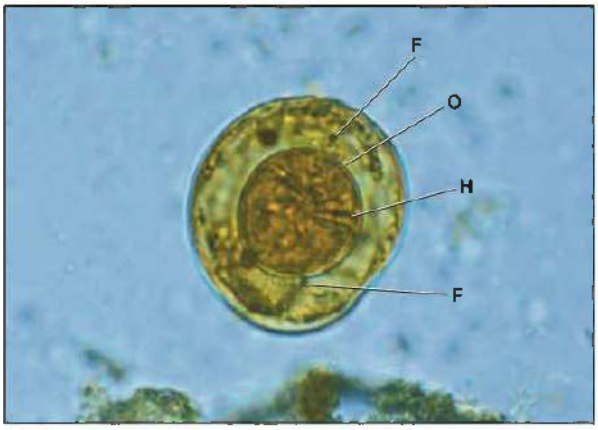
Hymenolepis nana (Figs. 12.56 and 12.57) is the dwarf tapeworm and is the most common cestode parasite of humans in the world. It is found in areas of high population density with poor sanitation. When eggs (Fig. 12.58) are ingested, the oncospheres (larvae retained with an egg) develop into juveniles in the lymphatics of intestinal villi. These juveniles are then released into the lumen within a week and attach to the mucosa to mature into adults. Infection may involve hundreds of worms, yet symptoms are usually absent or mild: diarrhea, nausea, loss of appetite, or abdominal pain. Eggs may reinfect the same host or pass out with the feces to infect a new host; no intermediate host is necessary. Eggs in the feces are used for identification, but proglottids are not because they are rarely passed. Treatment is with praziquantel and prevention requires improved sanitation.



12.56 ***Hymenolepis nana* Scolex** ◆ Adults gain a length of up to 10 cm, but are only 1 mm in width. The rostellum (R) is armed with up to 30 hooks (H).



12.57 ***Hymenolepis nana* Proglottids** ◆ *H. nana* is hermaphroditic, but in this specimen the numerous testes obscure most other structures.



12.58 ***Hymenolepis nana* Egg in a Fecal Sample (D'Antoni's Iodine Stain)** ◆ The *Hymenolepis nana* egg is 30 µm to 47 µm in diameter and has a thin shell. The oncosphere (O) is separated from the shell and contains six hooks (H). Another distinguishing feature is the presence of between four and eight filaments (F) arising from either end of the oncosphere.

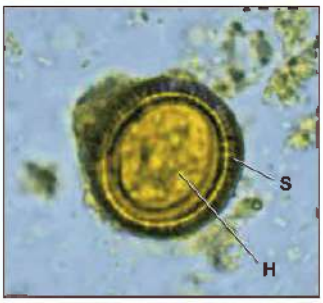
Taenia spp.

Two taeniid worms are important human pathogens. These are *Taenia saginata* (*Taeniarhynchus saginatus*), the beef tapeworm; and *Taenia solium*, the pork tapeworm. Both tapeworms are found worldwide and can grow to lengths of several meters and survive in human intestines for 25 years. *T. solium* may produce 1,000+ proglottids, each with 50,000 eggs, whereas *T. saginata* may produce 2,000+ proglottids, each with 100,000 eggs! Humans are the definitive host for both.

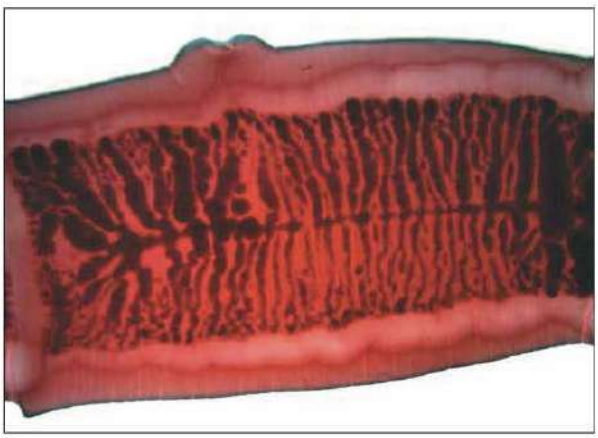
Cattle are the intermediate hosts of *T. saginata* and become infected when they ingest eggs or proglottids in fecally contaminated soil or water. Juveniles are dispersed throughout the body and infect various organs, but most importantly to the life cycle, skeletal muscle. As a result, humans who eat undercooked beef containing juvenile cysticerci become infected. In the presence of bile salts, cysticerci develop into adults and begin producing gravid proglottids in the small intestine within a few weeks. Symptoms of infection are usually mild nausea, diarrhea, abdominal pain, and headache. Diagnosis to species is impossible with only the eggs (Fig. 12.59); specific identification requires a scolex or gravid proglottid (Fig. 12.60). Treatment is with praziquantel or niclosamide. Finding a scolex in a fecal sample is considered a sure sign of successful treatment.

The *T. solium* life cycle is similar to *T. saginata*, but the host is pork, not beef, so human infection with the adult worm occurs when undercooked pork is eaten. Symptoms of infection are generally mild and involve the digestive tract. However, if a human ingests eggs, cysticerci develop and may be found in any tissue, including the eyes, brain, heart, liver, lungs, and skeletal muscle. Symptoms

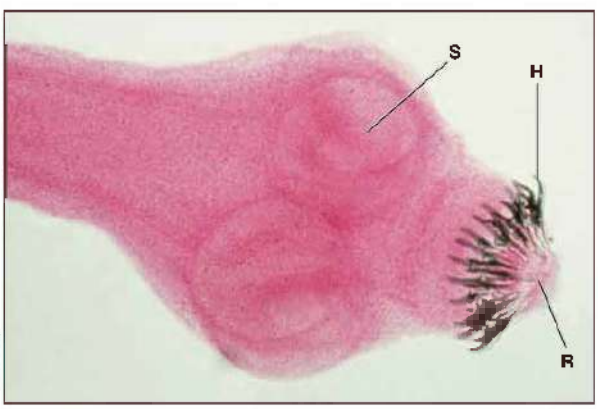
of cysticercosis depend on the tissue infected, but mostly they are not severe. However, infection of the brain can lead to seizures, and death of a cysticercus can produce a rapidly fatal inflammatory response. As with *T. saginata*, diagnosis to species is impossible with only the eggs; specific identification requires a scolex or gravid proglottid (Figs. 12.61 and 12.62). Treatment is the same as for *T. saginata*.



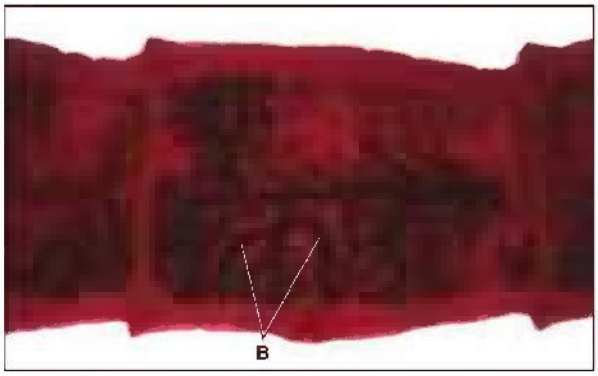
12.59 *Taenia* Egg in a Fecal Sample ♦ Taeniid eggs are distinctive looking enough to identify to genus, but not distinctive enough to speciate. Eggs are spherical and approximately 40 µm in diameter with a striated shell (S). The oncosphere contains six hooks (H).



12.60 *Taenia saginata* Proglottid ♦ The uterus of *Taenia saginata* proglottids consists of a central portion with 15 to 20 lateral branches (compare to the *T. solium* uterus in Fig. 12.62).



12.61 *Taenia solium* Scolex ♦ The *Taenia solium* scolex has two rings of hooks (H), four suckers (S), and a protruding rostellum (R).



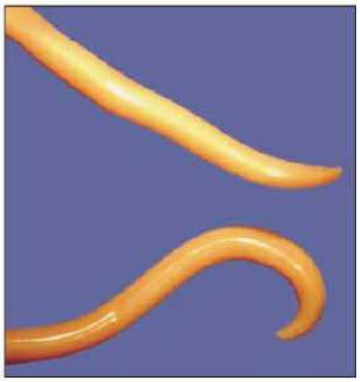
12.62 *Taenia solium* Proglottid ♦ The uterus of *Taenia solium* proglottids consists of a central portion with 7 to 13 lateral branches (B) (compare to the *T. saginata* uterus in Fig. 12.60).

Nematode Parasites Found in Clinical Specimens

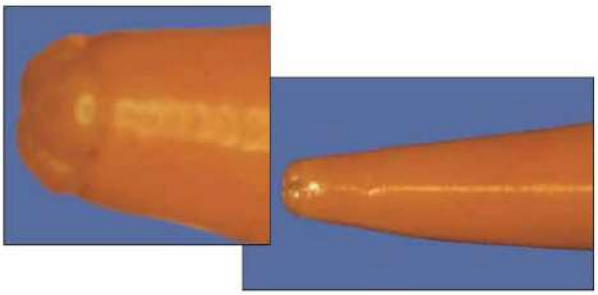
Free-living species in the phylum Nematoda, commonly called roundworms, are abundant in virtually all terrestrial and aquatic ecosystems. Parasitic species, which are our focus, equal free-living species in number with over 60 species being human parasites. Anatomically, nematodes have a complete digestive tract, a body cavity, true reproductive organs in separate sexes, and are unsegmented.

Ascaris lumbricoides

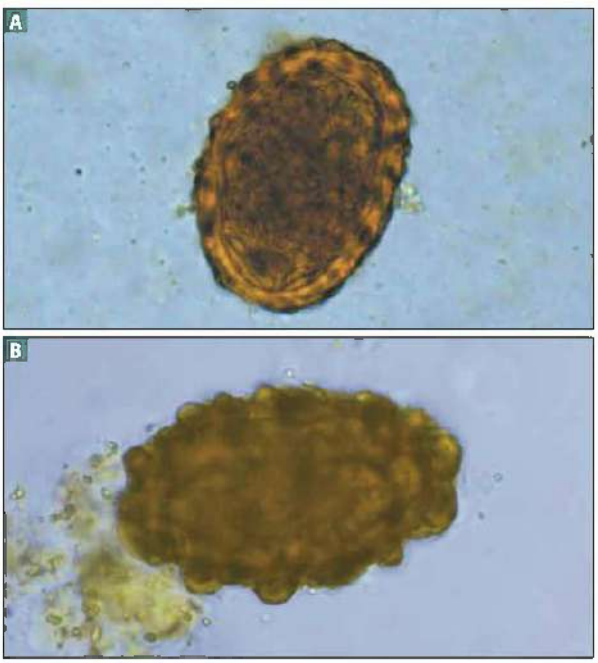
Ascaris lumbricoides (Figs. 12.63 and 12.64) is a large nematode—females may reach a length of 49 cm—and is most commonly found in tropical regions. Human infection occurs when eggs in fecally contaminated soil or food are ingested. Juveniles emerge in the intestine, pass through its wall, and then migrate to the lungs and other tissues. After a period of development in the lungs, juveniles move up the respiratory tree to the esophagus and are swallowed again. Adults then reside in the small intestine and produce eggs (Figs. 12.65), which are passed in the feces. There is no intermediate host. Patients are usually asymptomatic, but may experience varying degrees of intestinal obstruction and malnutrition, depending on the parasite load. Juvenile worms in the lungs can cause *Ascaris* pneumonia as a result of a hypersensitivity reaction. If secondary bacterial infections occur, the pneumonia can be fatal. Identification of an *Ascaris* infection is made by observing the eggs in feces. Several antihelmintic drugs are available for treatment and improved sanitation is effective in minimizing transmission.



12.63 *Ascaris lumbricoides* Adult Worms (Preserved Specimens) ◆ *A. lumbricoides* males (bottom) are shorter than females (up to 31 cm vs. 35 cm) and have a curved posterior.



12.64 *Ascaris lumbricoides* Anterior ◆ *A. lumbricoides* has a cylindrical shape with three prominent mouthparts (see inset).

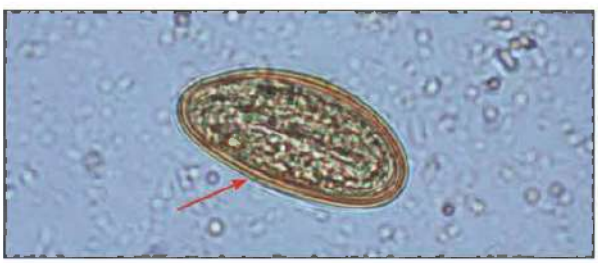


12.65 *Ascaris lumbricoides* Eggs in a Fecal Sample (D'Antoni's Iodine Stain) ◆ (A) Fertile *Ascaris lumbricoides* eggs are 55 µm to 75 µm long by 35 µm to 50 µm wide and are embryonated. Their surface is covered by small bumps called mammillations. (B) Infertile eggs are longer (up to 90 µm) than fertile eggs. There is no embryo inside.

Enterobius vermicularis

Enterobius vermicularis (Fig. 12.66) is the human pinworm. It is found worldwide and is especially prevalent among people in institutions, because conditions favor fecal–oral transmission of the eggs (Fig. 12.67). Poor sanitary habits of children make them especially prone to infecting others. Transmission may also involve eggs being carried on air currents and then inhaled by a susceptible host. After ingestion, eggs hatch in the duodenum and mature in the large intestine where the adults reside. Adult females emerge from the anus at night to lay thousands of eggs in the perianal region. About one-third of pinworm infections are asymptomatic; the other two-thirds usually do not produce serious symptoms. Diagnosis is made by identifying the eggs. Since eggs are laid externally, they are rarely found in feces. Instead, they are collected on cellophane tape from the perianal region and examined microscopically. Several antihelmintic drugs are available for treatment and improved personal hygiene is effective in minimizing transmission.

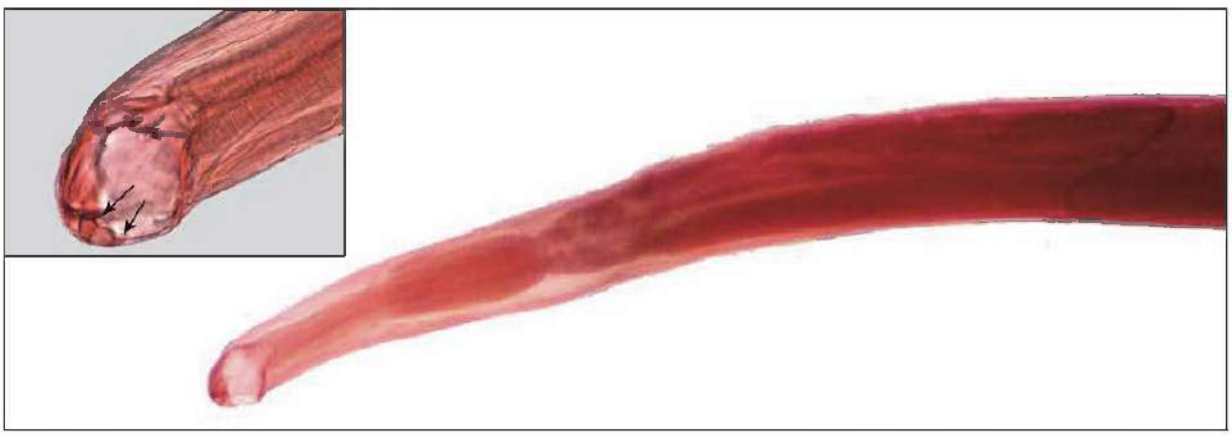
12.66 *Enterobius vermicularis* Adult Female (Preserved Specimen) ◆ Female pinworms are about 1 cm long and have a pointed tail (T) from which this group derives its common name—pinworm. Males are about half that size and have a hooked tail.



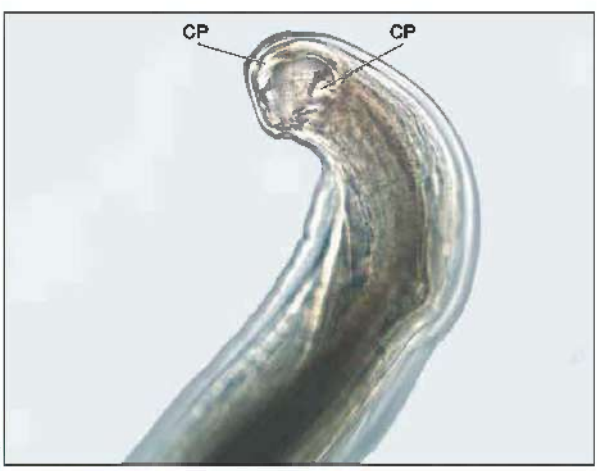
12.67 *Enterobius vermicularis* Egg in a Fecal Sample (D'Antoni's Iodine Stain) ◆ The eggs of *Enterobius vermicularis* are 50 µm to 60 µm long by 20 µm to 40 µm wide with one side flattened (arrow). They are usually embryonated in typical preparations.

Hookworms (*Ancylostoma duodenale* and *Necator americanus*)

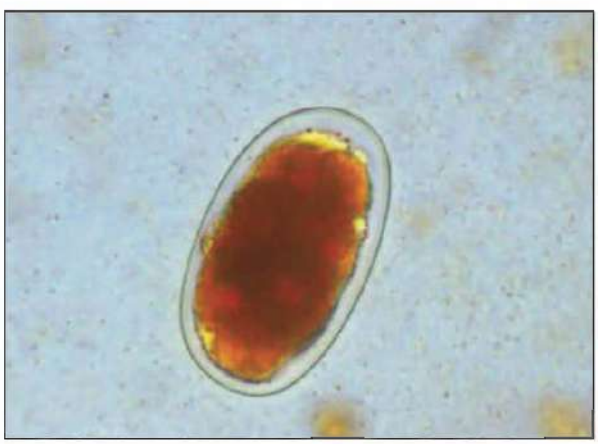
The hookworms, *Ancylostoma duodenale* (Fig. 12.68) and *Necator americanus* (Fig. 12.69), have very similar morphologies and life cycles, and the eggs are indistinguishable, so they are considered together here. Both are found in regions where warm, moist soil is present. Infection occurs when juveniles penetrate the skin, enter the blood and travel to the lungs. They penetrate the respiratory membrane and are carried up and out of the lungs by ciliary action to the pharynx, where they are swallowed. When they reach the small intestine, they attach and mature into adults that feed on blood and tissues of the host. Adults are rarely seen, because they remain attached to the intestinal mucosa. Eggs (Fig. 12.70) are passed in the feces and are diagnostic of infection. Hookworms produce immunosuppressant proteins that enhance their ability to survive in the host, but most infections are still asymptomatic. When symptoms appear, their severity is related to the parasite load. Severe symptoms of bloody diarrhea and iron-deficiency anemia are only seen in acute heavy or chronic infections. A rash may develop where the larva penetrated the skin and this site is susceptible to secondary bacterial infection. Treatment is available with a variety of antihelmintic drugs. Covering the skin (e.g., with footwear) and not frequenting areas suspected of soil contamination are successful preventative measures.



12.68 Anterior of *Ancylostoma duodenale* (Preserved Specimen) ◆ *Ancylostoma duodenale* is the Old World hookworm. Shown is the head with the mouth and thick-walled esophagus. The bend in the head gives this group its common name—hookworm. In the inset, the chitinous teeth (arrows) are visible (compare with Fig. 12.69).



12.69 *Necator americanus* Head (Preserved Specimen) ◆ This detail of *N. americanus*, the New World hookworm, shows the cutting plates (CP) that help to differentiate it from *A. duodenale* (compare with Fig. 12.68). Also notice the hooked head.



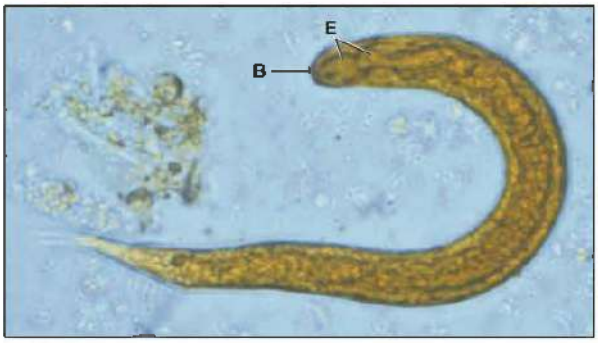
12.70 Hookworm Egg in a Fecal Sample (D'Antoni's Iodine Stain) ◆ Hookworm eggs are 55 µm to 75 µm long by 36 µm to 40 µm wide. They have a thin shell and contain a developing embryo (seen here at about the 16 cell stage) that is separated from the shell when seen in fecal samples.

Strongyloides stercoralis

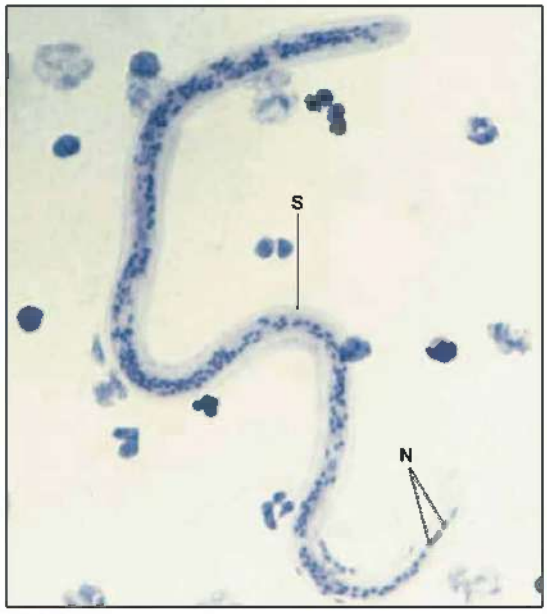
Strongyloides stercoralis is the intestinal threadworm. Infection occurs when infective juveniles from fecally contaminated soil penetrate the skin. The juveniles then are carried by the blood to the lungs where they migrate to the pharynx and are swallowed. In the intestines, they develop into parthenogenetic females (*S. stercoralis* does not produce parasitic adult male worms) that burrow into the intestinal mucosa. Each day, females release a few dozen eggs that develop into larvae (Fig. 12.71) before they are passed in the feces or reinfect the intestines (autoinfection). Once passed, larvae may become infective and complete the life cycle or they may follow a developmental path that produces free-living adult males and females. Free-living adults produce infective juveniles and the cycle (with sexual reproduction) is completed. Symptoms of infection may be itching or secondary bacterial infection at the entry site of infective larvae, a cough and burning of the chest during the pulmonary phase, and abdominal pain and perhaps septicemia during the intestinal phase. Diagnosis is by finding rhabditiform larvae (rarely eggs) in fresh fecal samples. Failing that (because of their relatively small numbers), an ELISA is also available. Ivermectin is used for treatment of most infections and may be supplemented with antibiotics to prevent secondary bacterial infections.

Wuchereria bancrofti

Wuchereria bancrofti is the most commonly identified filarial worm. It is found in tropical and subtropical regions worldwide and causes lymphatic filariasis. Humans are the definitive host and several mosquito species are the intermediate host. Infection occurs when they transfer infective juveniles during a blood meal. Once in the host, worms migrate into the large lymphatic vessels of the lower body where they mature and may reside for years. Adults are found in coiled bunches and the females release microfilariae (Fig. 12.72) by the thousands. Microfilariae enter the blood and circulate there, often with a daily periodicity—most abundant at night when the mosquito vector is active and hidden away in lung capillaries during the day when it is hot. Some infections are asymptomatic, whereas others result in acute inflammation of lymphatics associated with fever, chills, tenderness, and toxemia. In the most serious cases, obstruction of lymphatic vessels occurs and results in elephantiasis, a disease caused by accumulation of lymph fluid in the tissues, an accumulation of fibrous connective tissue, and a thickening of the skin. Diagnosis of infection is made by identifying microfilariae in blood smears or in some cases, ultrasound. Treatment of active infection is with the antihelmintic drug diethylcarbamazine (DEC). Protecting oneself against mosquito bites is an effective means of prevention, though not always a practical or attainable one.



12.71 *Strongyloides stercoralis* Rhabditiform Larva in Feces (D'Antoni's Iodine Stain) ◆ Rhabditiform larvae are noninfective, but develop into the infective filariform larvae. The name "rhabditiform" refers to the esophagus shape (E), which has a constriction within it. They may be distinguished from hookworm larvae (which are rarely in feces) by their short buccal cavity (B).



12.72 *Wuchereria bancrofti* Microfilaria in a Blood Sample ◆ The microfilariae of *Wuchereria bancrofti* can be distinguished from others in the blood by the sheath (S) and the single column of nuclei (N) not extending to the tip of the tail.

Application

Medical microbiologists rely heavily on microscopic examination of patient samples (e.g., blood and feces) to identify parasitic worms, either through directly seeing the worm or more frequently, seeing distinctive worm eggs.

In This Exercise

Today you will examine prepared slides of parasitic worms as an introduction to their structural diversity and relate what you see to their life cycles and ability to cause disease.

✓ Materials

□ Prepared slides of:

- *Ascaris lumbricoides* eggs in a fecal smear
- *Clonorchis sinensis* in a fecal smear
- *Dipylidium caninum* eggs in a fecal smear
- *Echinococcus granulosus* hydatid cyst in section
- *Enterobius vermicularis* eggs in a fecal smear
- Hookworm (*Ancylostoma duodenale* or *Necator americanus*) eggs in a fecal smear
- *Hymenolepis nana* eggs in a fecal smear
- *Paragonimus westermani* eggs in a fecal smear
- *Schistosoma mansoni* eggs in a fecal smear
- *Strongyloides stercoralis* rhabditiform larvae in a fecal smear
- *Taenia solium* proglottid (whole mount)
- *Taenia solium* scolex (whole mount)
- *Taenia* spp. eggs in a fecal smear
- *Wuchereria bancrofti* microfilariae in a blood smear

Procedure

1 Obtain prepared slides of helminth specimens in fecal smears and other tissues. Scanning on low power (10× objective) is best for most preparations. Then move to high dry or oil immersion to see detail. If the coverslip does not get in the way, observe from the side as you rotate the nosepiece and do not force the oil lens into position if it looks like it will hit the coverslip. Most egg specimens are in fecal smears, so there will be a lot of other material on the slide besides the eggs. You must search carefully and with patience.

- *Clonorchis sinensis* in a fecal smear. Identify eggs and operculum.
- *Paragonimus westermani* eggs in a fecal smear. Identify eggs, operculum, and thickened abopercular wall.
- *Schistosoma mansoni* eggs in a fecal smear. Identify eggs and spine.
- *Dipylidium caninum* eggs in a fecal smear. Identify egg packets, eggs, and oncospheres.
- *Echinococcus granulosus* hydatid cyst in section. Identify fibrous, acellular, and germinal layers of hydatid cyst, brood capsule membrane, protoscolices, and hooks.
- *Hymenolepis nana* eggs in a fecal smear. Identify eggs, oncospheres, filaments, and hooks.
- *Taenia* spp. eggs in a fecal smear. Identify spherical eggs, striated shells, oncospheres, and six hooks.
- *Taenia solium* scolex (whole mount). Identify scolex, rostellum, two rings of hooks, and suckers.
- *Taenia solium* proglottid (whole mount). Identify proglottid(s) and uterus with branches
- *Ascaris lumbricoides* eggs in a fecal smear. Identify fertile eggs with embryos, infertile eggs, and mammillations.
- *Enterobius vermicularis* eggs in a fecal smear. Identify elongated eggs with flattened side and embryos.
- Hookworm (*Ancylostoma duodenale* or *Necator americanus*) eggs in a fecal smear. Identify eggs, embryos, and thin shells.
- *Strongyloides stercoralis* rhabditiform larva in a fecal smear. Identify buccal cavity and constricted esophagus.
- *Wuchereria bancrofti* microfilariae in a blood smear. Identify microfilariae, sheath, single column of nuclei in tail not extending to its end, and red blood cells.

2 Carefully sketch and label one or two representative examples in the spaces provided on the data sheet, page 823, and measure dimensions. Be able to identify each species if shown an unlabeled specimen.

References

Ash, Lawrence R. and Thomas C. Orihel. Chaps. 15 and 16 in *Parasites: A Guide to Laboratory Procedures and Identification.* Chicago: American Society for Clinical Pathology Press, 1991.

Fink, Doran L and Thomas B. Nutman. Chap. 142 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock eds. Washington, DC: ASM Press, 2011.

Garcia, Hector H., Juan A. Jimenez, and Hermes Escalante. Chap. 143 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Garcia, Lynne Shore. Chaps. 10, 12, 13, 14, 16, and 17 in *Diagnostic Medical Parasitology,* 4th ed. Washington, DC: ASM Press, 2001.

Jones, Malcolm K., Jennifer Keiser, and Donald P.McManus. Chap. 144 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Koneman, Elmer W., Stephen D. Allen, William M. Janda, Paul C. Schreckenberger, and Washington C. Winn, Jr. Chap. 20 in *Color Atlas and Textbook of Diagnostic Microbiology,* 5th ed. Philadelphia: J. B. Lippincott Company, 1997.

Reece, Jane B., Lisa A. Urry, Michael L. Cain, Steven A. Wasserman, Peter V. Minorsky, and Robert B. Jackson. Chap. 33 in *Campbell Biology* 10th ed. Boston: Pearson, 2014.

Roberts, Larry S. and John Janovy, Jr. *Foundations of Parasitology,* 5th ed. Dubuque, IA: Wm. C. Brown Publishers, 1996.

Sheorey, Harsha, Beverley-Ann Biggs, and Peter Traynor. Chap. 141 in *Manual of Clinical Microbiology,* 10th ed. James Versalovic, Karen C. Carroll, Guido Funke, James H. Jorgensen, Marie Louise Landry, and David W. Warnock, eds. Washington, DC: ASM Press, 2011.

Tille, Patricia M. Chaps. 51, 53, 54, 55, and 57 in *Bailey & Scott's Diagnostic Microbiology,* 13th ed. St. Louis, MO: Mosby, 2014.

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 12-4

Parasitic Helminths

OBSERVATIONS AND INTERPRETATIONS

1 Carefully sketch and label your observations of one or two examples of the assigned helminths in the circles provided below.

Trematodes

Clonorchis sinensis
egg

(× _____)

Dimensions _____

Paragonimus westermani
egg

(× _____)

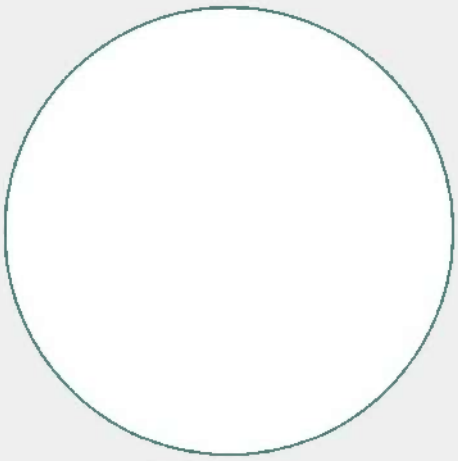
Dimensions _____

Schistosoma mansoni
egg

(× _____)

Dimensions _____

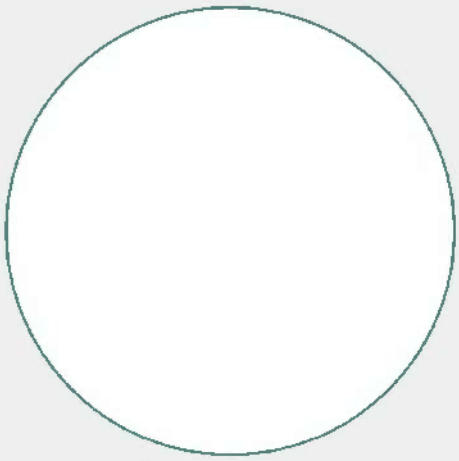
Cestodes



Dipylidium caninum
egg packet

(× _____)

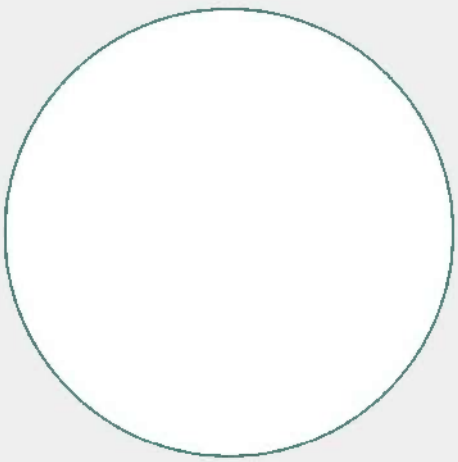
Dimensions _____



Echinococcus granulosus
protoscolices in a hydatid cyst

(× _____)

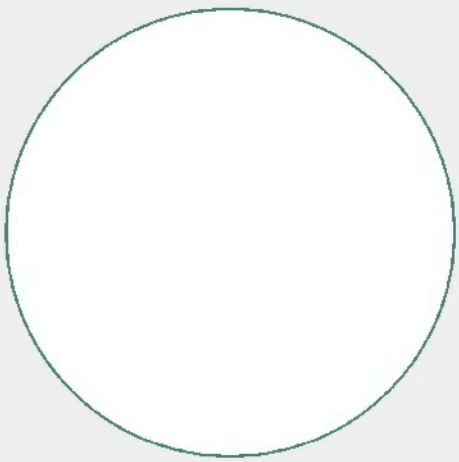
Dimensions _____



Hymenolepis nana
egg with oncosphere and filaments

(× _____)

Dimensions _____



Taenia spp.
egg

(× _____)

Dimensions _____

12

Name _____

Date _____

Lab Section _____

I was present and performed this exercise (initials) _____

DATA SHEET 12-4

(continued)

Taenia solium
scolex

(× _____)

Dimensions _____

Taenia solium
proglottid

(× _____)

Dimensions _____

Average number of uterine branches _____

Nematodes

Ascaris lumbricoides
eggs with mammillations

(× _____)

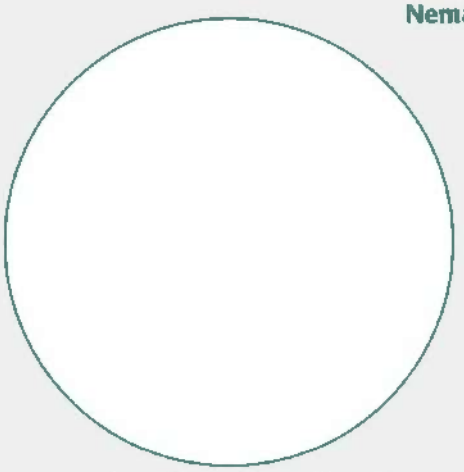
Dimensions _____

Enterobius vermicularis
egg

(× _____)

Dimensions _____

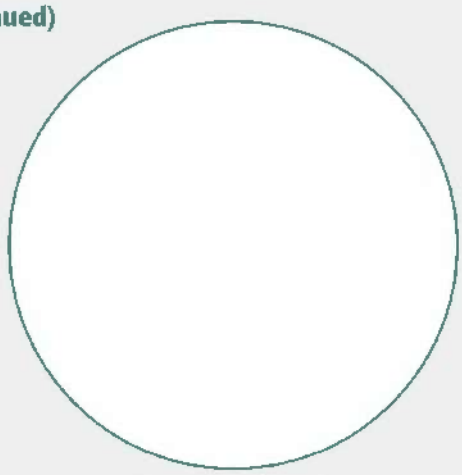
Nematodes (continued)



Hookworm (*Ancylostoma* or *Necator*)
egg

(× _____)

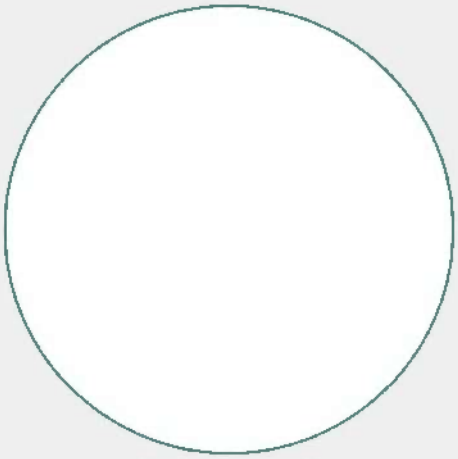
Dimensions _____



Strongyloides stercoralis
rhabditiform larva

(× _____)

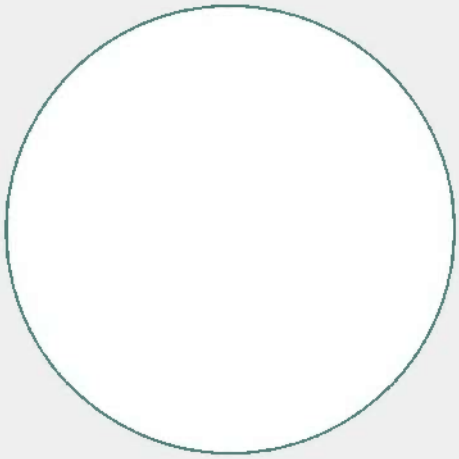
Dimensions _____



Wuchereria bancrofti
microfilariae in a blood smear

(× _____)

Dimensions _____



_____

_____

(× _____)

Dimensions _____

12

APPENDIX A

Biochemical Pathways

So much of what is done in microbiology relies on an understanding of basic biochemical pathways. It is not as important to memorize them (although, with exposure they will become second nature) as it is to understand their importance in metabolism and to interpret diagrams of them when available. The following discussion is provided so you can see how the various biochemical tests presented in this manual fit into the overall scheme of cellular chemistry.

Oxidation of Glucose: Glycolysis, Entner-Doudoroff, and Pentose-Phosphate Pathways

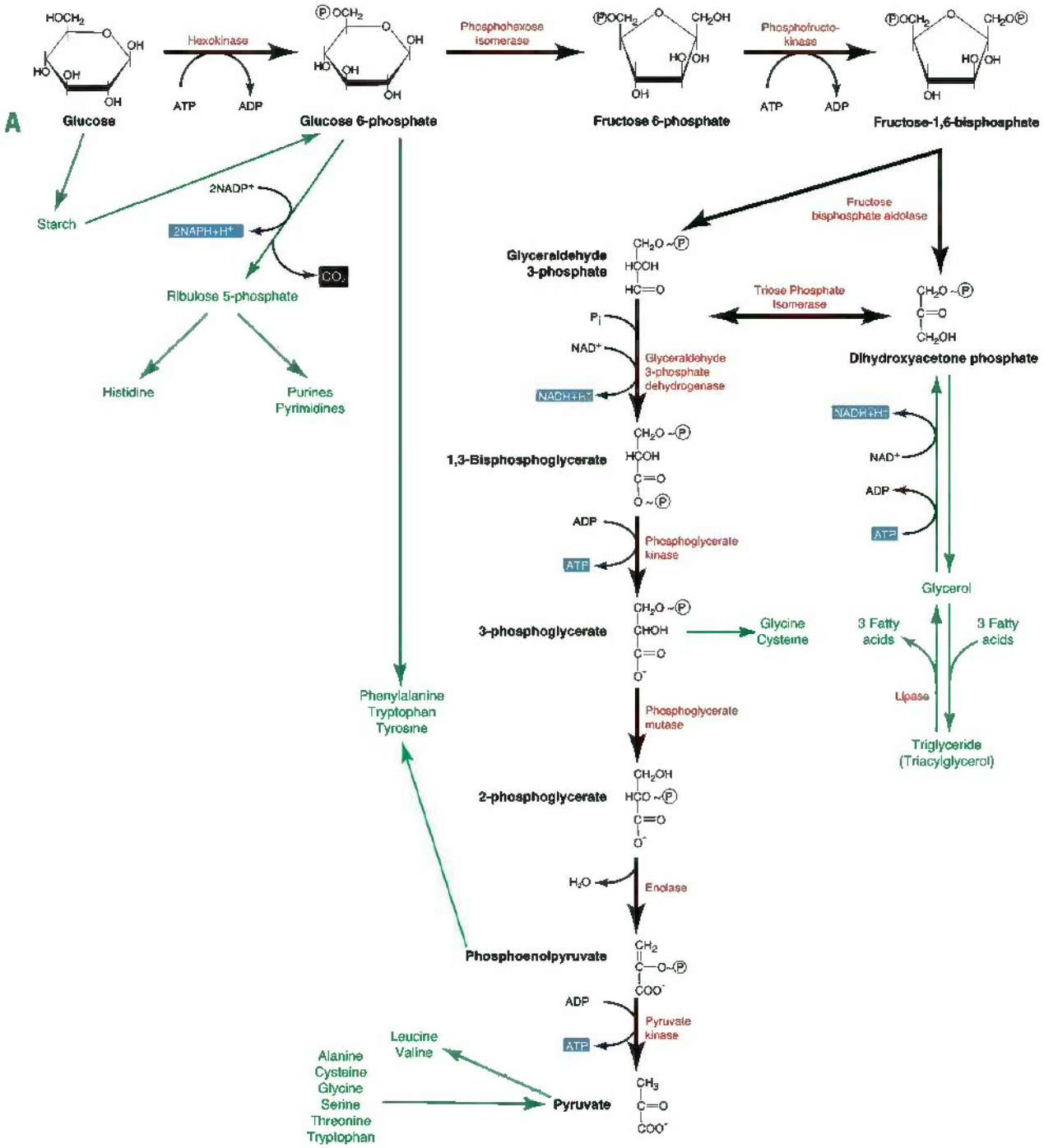
Most organisms use glycolysis, also known as the Embden-Meyerhof-Parnas pathway, (Fig. A.1) in energy metabolism. It performs the stepwise disassembly of glucose into two pyruvates, releasing some of its energy and electrons in the process. The exergonic (energy-releasing) reactions are associated with ATP synthesis by a process called substrate phosphorylation. Although a total of four ATPs are produced per glucose in glycolysis, two ATPs are hydrolyzed early in the pathway, leaving a net production of two ATPs per glucose. In one glycolytic reaction, the loss of an electron pair (oxidation) from a 3-carbon intermediate occurs simultaneously with the reduction of NAD^+ to $NADH + H^+$. The $NADH + H^+$ then may be oxidized in an electron transport chain or a fermentation pathway, depending on the organism and the environmental conditions. The former yields ATP and the latter generally does not. In summary, each glucose oxidized in glycolysis yields two pyruvates, $2\ NADH + 2\ H^+$, and a net of 2 ATPs (Table A-1).

Although the intermediates of glycolysis are carbohydrates, many are entry points for amino acid, lipid, and nucleotide catabolism. Many glycolytic intermediates also are a source of carbon skeletons for the synthesis of these other biochemicals. Some of these are shown in Figure A.1. *Note*: For clarity, many details have been omitted from these other pathways in Figure A.1. Single arrows may represent several reactions, and other carbon compounds not illustrated may be required to complete a particular reaction.

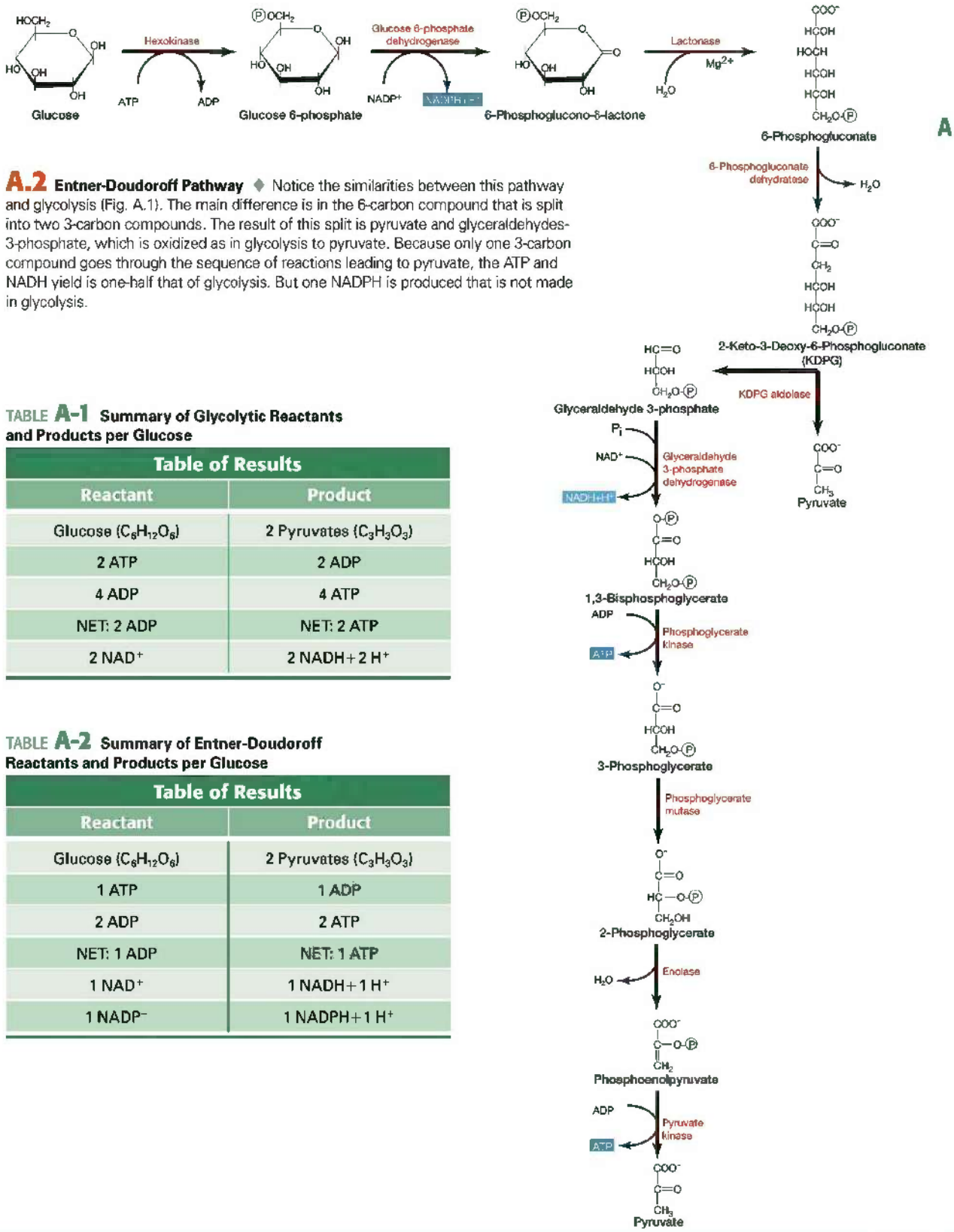
The Entner-Doudoroff pathway (Fig. A.2) is an alternative means of degrading glucose into two pyruvates. This pathway is found exclusively among prokaryotes (e.g., *Pseudomonas* and *E. coli*, as well as other Gram negatives and certain Archaeans). It allows utilization of a different category of sugars (aldonic acids) than glycolysis and therefore improves the range of resources available to the organism. It is less efficient than glycolysis because only one ATP is phosphorylated and only one NADH is produced. Table A-2 summarizes this pathway.

The pentose-phosphate pathway is a complex set of cyclic reactions that provides a mechanism for producing 5-carbon sugars (pentoses) from 6-carbon sugars (hexoses). Pentose sugars are used in ribonucleotides and deoxyribonucleotides, as well as being precursors to aromatic amino acids. Further, this pathway produces NADPH, which is used as an electron donor in anabolic pathways. Unlike NADH, produced in glycolysis and Entner-Doudoroff, NADPH is not used as an electron donor in an electron transport chain for oxidative phosphorylation of ADP.

The pentose-phosphate reactants and products are listed in Table A-3, and the overall path is shown in Figure A.3. To completely oxidize one hexose to $6CO_2$, a total of six hexoses must enter the cycle as glucose-6-phosphate and follow one of three different routes (notice the symmetry of pathways as drawn). Notice in Figure A.3 that each hexose loses a CO_2 upon entry into the cycle, but at the end, five hexoses are produced. Thus, the net reaction is one hexose being oxidized to $6CO_2$. Notice also the reactions that transfer 2-carbon and 3-carbon fragments between the 5-carbon intermediates. Transketolase catalyzes the 2-carbon transfer, whereas transaldolase catalyzes the 3-carbon transfer.



A.1 Glycolysis and Associated Pathways ◆ The names of glycolytic intermediates are printed in black ink; the enzyme names are in red. Reducing power (in the form of NADH + H^+) and ATP are highlighted in blue. Compounds in pathways that intersect with glycolytic intermediates are in green. The major key to getting product yields correct is to recognize that both C_3 compounds (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate) produced from splitting fructose 1,6-bisphosphate can pass through the remainder of the pathway because of the triose phosphate isomerase reaction. The conversion of each into pyruvate results in the formation of 2 ATPs and 1 NADH + H^+ (Table A-1).



A.2 Entner-Doudoroff Pathway ♦ Notice the similarities between this pathway and glycolysis (Fig. A.1). The main difference is in the 6-carbon compound that is split into two 3-carbon compounds. The result of this split is pyruvate and glyceraldehydes-3-phosphate, which is oxidized as in glycolysis to pyruvate. Because only one 3-carbon compound goes through the sequence of reactions leading to pyruvate, the ATP and NADH yield is one-half that of glycolysis. But one NADPH is produced that is not made in glycolysis.

TABLE A-1 Summary of Glycolytic Reactants and Products per Glucose

Table of Results	
Reactant	**Product**
Glucose ($C_6H_{12}O_6$)	2 Pyruvates ($C_3H_3O_3$)
2 ATP	2 ADP
4 ADP	4 ATP
NET: 2 ADP	NET: 2 ATP
2 NAD^+	2 NADH + 2 H^+

TABLE A-2 Summary of Entner-Doudoroff Reactants and Products per Glucose

Table of Results	
Reactant	**Product**
Glucose ($C_6H_{12}O_6$)	2 Pyruvates ($C_3H_3O_3$)
1 ATP	1 ADP
2 ADP	2 ATP
NET: 1 ADP	NET: 1 ATP
1 NAD^+	1 NADH + 1 H^+
1 $NADP^-$	1 NADPH + 1 H^+

TABLE A-3 Summary of Pentose-Phosphate Reactants and Products per Glucose-6-phosphate

Table of Results	
Reactant	**Product**
Glucose-6-phosphate (C_6)	$6CO_2 + 1\ P_i$
$12\ NADP^+$	$12\ NADPH + 12\ H^+$

A

Alternatively, the 5-carbon intermediates can be redirected into pathways for synthesis of aromatic amino acids and nucleotides (not shown).

Oxidation of Pyruvate: The Citric Acid Cycle and Fermentation

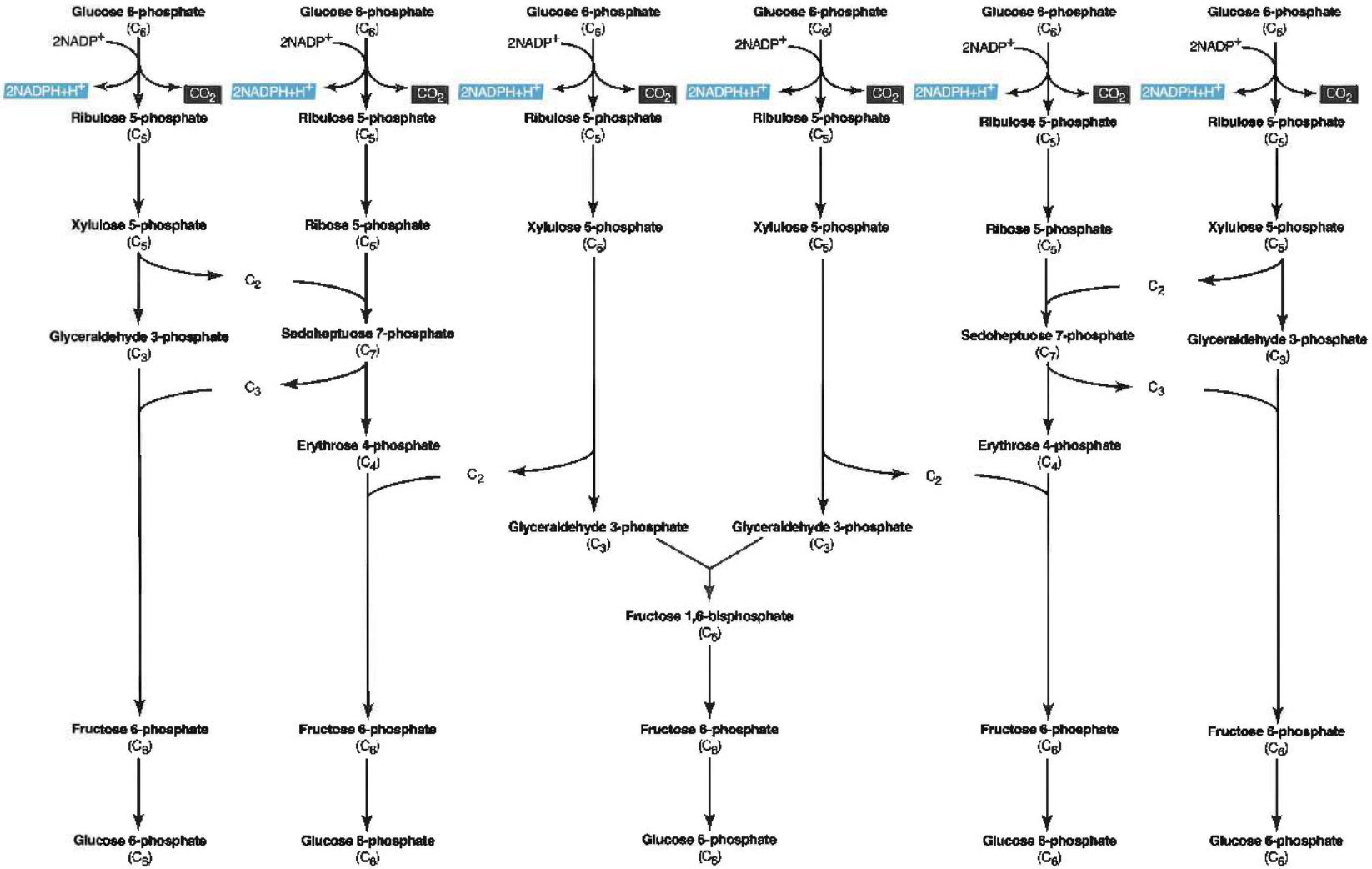
Pyruvate represents a major crossroads in metabolism. Some organisms are able to further disassemble the pyruvates produced in glycolysis and Entner-Doudoroff and make more ATP and $NADH + H^+$ glucose-6-phosphate in the citric acid cycle. Other organisms simply reduce the pyruvates with electrons from $NADH + H^+$ without further energy production in fermentation.

The citric acid cycle is a major metabolic pathway used in energy production by organisms that respire aerobically or anaerobically (Fig. A.4). Pyruvate produced in glycolysis or other pathways is first converted to acetyl-coenzyme A during the entry step (also known as the intermediate, or gateway step). Acetyl-CoA enters the citric acid cycle through a condensation reaction with oxaloacetate. Products for each pyruvate that enters the cycle via the entry step are: 3 CO_2, 4 $NADH + H^+$, 1 $FADH_2$, and 1 GTP. (Because two pyruvates are made per glucose, these numbers are doubled in Table A-4). The energy released from oxidation of reduced coenzymes ($NADH + H^+$ and $FADH_2$) in an electron transport chain is then used to make ATP. ATP yields are summarized in Table A-5.

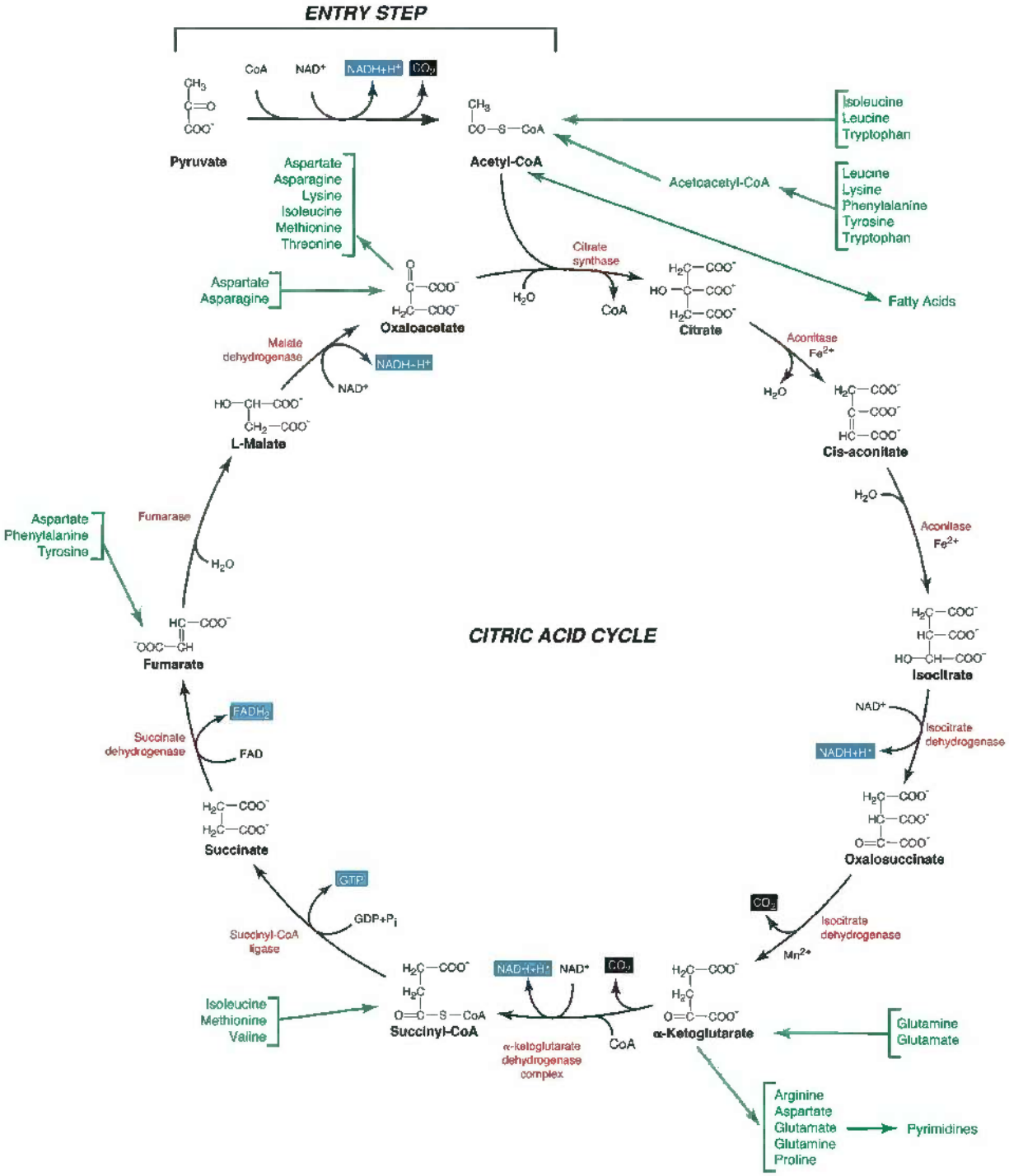
Like glycolysis, many of the citric acid cycle's intermediates are entry points for amino acid, nucleotide, and lipid catabolism, as well as a source of carbon skeletons for synthesis of the same compounds. These pathways are shown, but details have been omitted. Single arrows may represent several reactions, and other carbon compounds, not illustrated, may be required to complete a given reaction.

Figure A.5 illustrates some major fermentation pathways exhibited by microbes (though no single organism is capable of all of them). Pyruvate (shown in the blue box) is typically the starting point for each. End products of fermentation are shown in red. Fermentation allows some cells living under anaerobic conditions to oxidize reduced coenzymes (such as $NADH + H^+$ and shown in blue) generated during glycolysis or other pathways. Some bacteria (aerotolerant anaerobes) rely solely on fermentation and do not use oxygen even if it is available. Table A-6 summarizes major fermentations and some representative organisms that perform each.

Notice that fermentation end products typically fall into three categories: acid, gas, or an organic solvent (an alcohol or a ketone). The specific fermentation performed is the result of the enzymes present in a species and often is used as a basis of classification.



A.3 Pentose-Phosphate Cycle ◆ For every six glucose-6-phosphates that enter and complete the cycle, $6CO_2$ and 12 $NADPH + H^+$ are produced. Some of the 5-carbon intermediates, however, may be redirected into synthesis of aromatic amino acids and nucleotides. If the cycle is performed as shown, 36 carbons enter as six glucose 6-phosphates ($6 \times C_6 = 36C$). Six CO_2 are immediately lost, leaving a total of 30C to get shuffled around by the remaining reactions to form five glucose-6-phosphates ($5 \times C_6 = 30C$).



A.4 Entry Step and Citric Acid Cycle ◆ The names of intermediates are printed in black ink; enzymes are in red. Reducing power (in the form of $NADH + H^+$ and $FADH_2$) and GTP are highlighted in blue. CO_2 produced from the oxidation of carbon is highlighted in black. Compounds in pathways that intersect with the citric acid cycle are in green.

TABLE A-4 Summary of Reactants and Products per Glucose in the Entry Step and the Citric Acid Cycle

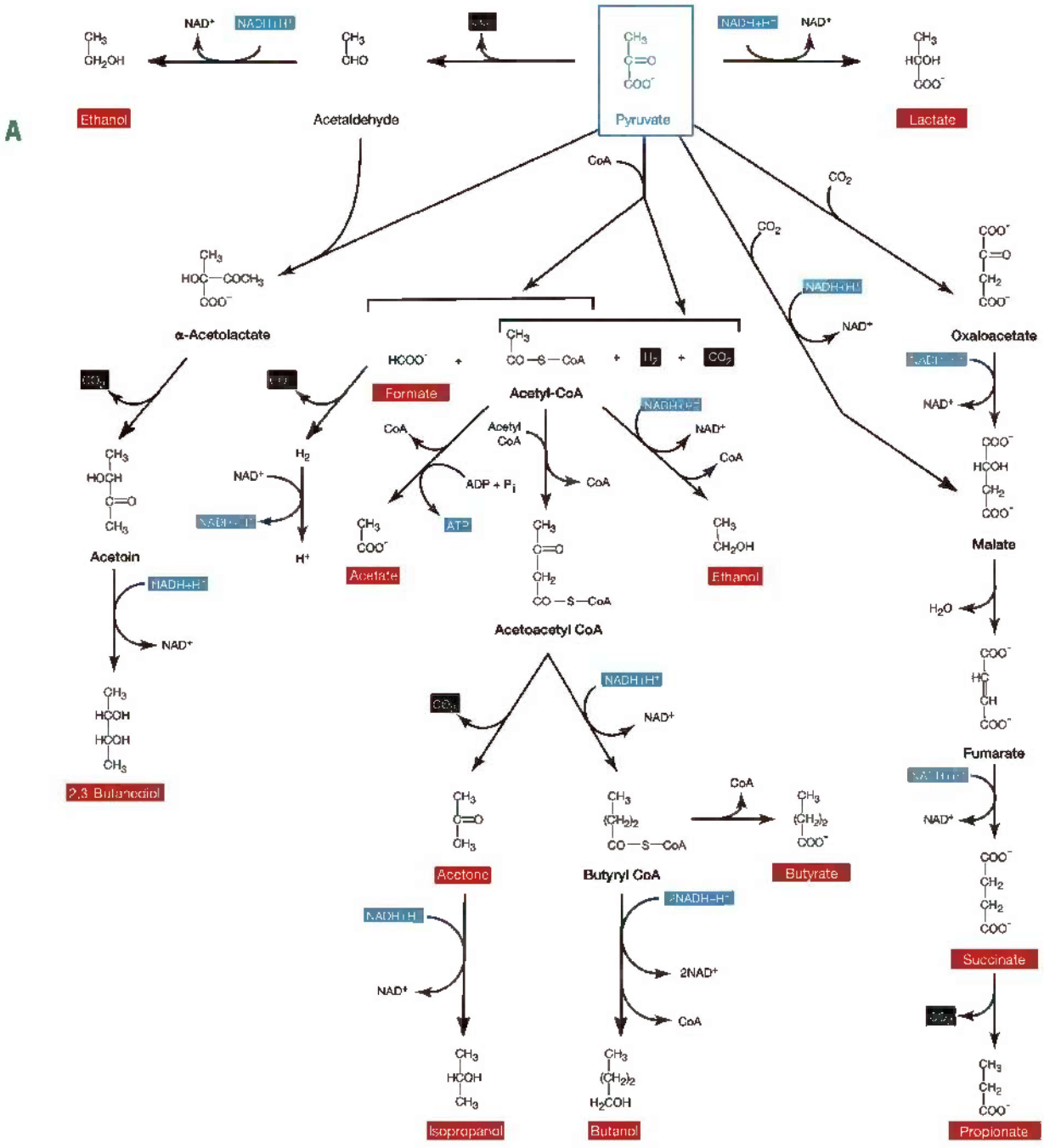
Entry Step		Citric Acid Cycle	
Reactant	Product	Reactant	Product
2 Pyruvates	2 Acetyl CoA + 2 CO_2	2 Acetyl CoA	4 CO_2
2 Coenzyme A			2 Coenzyme A
2 NAD^+	2 NADH+2H^+	6 NAD^+	6 NADH+6H^+
		2 GDP+2 P_i (= 2 ADP+2 P_i)	2 GTP (= 2 ATP)
		2 FAD	2 $FADH_2$

TABLE A-5 ATP Yields from Complete Oxidation of Glucose to CO_2 by a Prokaryote Using Glycolysis, Entry Step, and the Citric Acid Cycle with O_2 as the Final Electron Acceptor

Compound	Number Produced	ATP Value	Total ATPs per Glucose
NADH+H^+	10	3	30
$FADH_2$	2	2	4
ATP (by substrate phosphorylation)	4		4

TABLE A-6 Major Fermentations, Their End-Products, and Some Organisms That Perform Them

Fermentation	Major End Products	Representative Organisms
Alcoholic fermentation	Ethanol and CO_2	*Saccharomyces cerevisiae*
Homofermentation	Lactate	*Streptococcus* and some *Lactobacillus*
Heterofermentation	Lactate, ethanol, and acetate	*Streptococcus, Leuconostoc,* and *Lactobacillus*
Mixed acid fermentation	Acetate, formate, succinate, CO_2, H_2, and ethanol	*Escherichia, Salmonella, Klebsiella,* and *Shigella*
2,3-Butanediol fermentation	2,3-Butanediol	*Enterobacter, Serratia,* and *Erwinia*
Butyrate/butanol fermentation	Butanol, butyrate, acetone, and isopropanol	*Clostridium, Butyrivibrio,* and some *Bacillus*
Propionic acid fermentation	Propionate, acetate and CO_2	*Propionibacterium, Veillonella,* and some *Clostridium*



A.5 Sampling of Fermentation Pathways ♦ Note that all pathways start with pyruvate, have a step(s) where NADH + H^+ (in blue) is oxidized to NAD+, and produce end-products falling into one of three categories: acid, gas, or alcohol.

APPENDIX B

Miscellaneous Transfer Methods

Following are instructions for transfer methods that are performed less routinely than those in Exercise 1-3.

Transfers Using a Sterile Cotton Swab

A sterile swab generally is used to obtain a sample from a primary source—either a patient or an environmental site. Occasionally, swabs are used to transfer pure cultures. Sterile swabs may be dry, or they may be in sterile water, depending on the source of the sample. In either case, care must be taken not to contaminate the swab by unintentionally touching other surfaces with it. Your instructor may provide specific instructions on collection of samples from sources other than the ones below.

Obtaining a Sample from a Patient's Throat with a Swab

1. **Use BSL-2 precautions throughout.** Wear a lab coat, gloves, and eye protection.
2. Use a sterile tongue depressor and swab prepared in sterile water to obtain a sample from the throat. Have the patient open his/her mouth, and then gently press down on the tongue (Fig. B.1).
3. With the swab in your dominant hand, carefully sample the patient's throat with a swirling motion. Touching other parts of the oral cavity is likely to cause contamination. Also, avoid touching the soft palate or it may initiate a gag reflex!
4. Transfer the sample to an appropriate plated medium as quickly as possible. Use the swab to begin a quadrant streak (Exercise 1-4, Fig. 1.38). Properly dispose of the swab according to your lab's protocol, and then complete the streaking with your loop.
5. Label the plate with the sample source, date, and medium.

B.1 Taking a Throat Sample ◆ Use a sterile tongue depressor and swab to obtain a sample from a patient's throat. Be careful not to touch other parts of the oral cavity or the sample will get contaminated. Transfer the sample to a sterile medium as soon as possible.

6. If plating is to be done at a later time, place the swab in an appropriate sterile container (such as a sterile, capped test tube).
7. Incubate the plate in an inverted position at the appropriate temperature for the designated time.

Obtaining an Environmental Sample with a Cotton Swab

1. **Use BSL-2 precautions throughout.** Wear a lab coat, gloves, and eye protection.
2. Use a sterile swab prepared in sterile water to obtain a sample from an environmental source.
3. Rotate the swab to collect from the area to be sampled (Fig. B.2).
4. Transfer the sample to an appropriate plated medium as quickly as possible. Depending on the

B

B.2 Taking an Environmental Sample ◆ Use a spinning motion of a sterile swab to sample inanimate objects in the environment. The swab may be placed in a sterile test tube until it is convenient to transfer the sample to a growth medium.

anticipated cell density, use the swab to perform a zig-zag streak pattern (Exercise 1-4, Fig. 1.37) or to begin a quadrant streak with a disposable loop (Exercise 1-4, Fig. 1.38). Properly dispose of the swab and loop (if used) according to your lab's protocol.

5 Label the plate with the sample source, date, and medium.

6 If plating is to be done at a later time, place the swab in an appropriate sterile container (such as a sterile, capped test tube).

7 Incubate the plate in an inverted position at the appropriate temperature for the designated time.

Stab Inoculation of Agar Tubes Using an Inoculating Needle

Stab inoculations of agar tubes are used for several types of differential media (usually to examine growth under anaerobic conditions or to observe motility). A stab is *not* used to produce a culture of microbes for transfer to another medium.

1 Label the tube with your name, date, and organism or sample source.

2 Obtain the specimen with a sterile inoculating needle.

3 Remove the cap of the sterile medium with the little finger of your inoculating needle hand, and hold it there.

4 Flame the tube by quickly passing it through the Bunsen burner flame a couple of times. Keep your needle hand still.

5 Hold the open tube on an angle to minimize airborne contamination. Keep your needle hand still.

6 Carefully move the agar tube over the needle wire (Fig. B.3). Insert the needle into the agar to about 1 cm from the bottom.

7 Withdraw the tube carefully so the needle leaves along the same path it entered. (When removing the tube, be especially careful not to catch the needle tip on the tube lip. This springing action of the needle creates bacterial aerosols.)

8 Flame the tube lip as before. Keep your needle hand still.

9 Keeping the needle hand still (remember, it has growth on it), move the tube to replace its cap.

10 Incinerate the needle from base to tip to minimize aerosol production. **If transferring a BSL-2 organism, use a bacterial incinerator.**

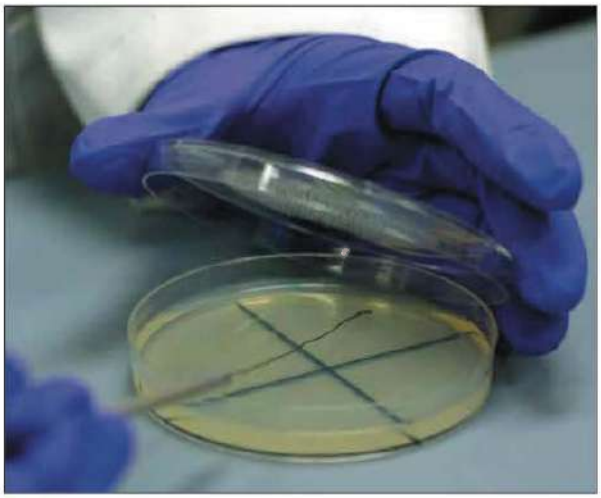
11 Incubate at the appropriate temperature for the assigned time.

B.3 Agar Deep Stab ◆ Use the inoculating needle to stab the agar to a depth about 1 cm from the bottom (tip of red arrow). It is generally desirable to remove the needle along the original stab line and not create a new one. Upon completion, sterilize the needle.

Spot Inoculation of an Agar Plate

Sometimes an agar plate may be used to grow several different specimens at once. This is a typical practice with plated *differential* media (i.e., media designed to differentiate organisms based on growth characteristics). Prior to beginning the transfer, divide the plate into as many as four sectors, using a marking pen. (Some plates already have marks on the base for this purpose.) Then each may be inoculated with a different organism. Inoculation involves touching the loop to the agar surface once so growth is restricted to a single spot—hence the name "spot inoculation."

1. Label the base of the plate with your name, date, medium, and organism(s) inoculated.
2. With a marking pen, divide the base of the plate into the desired number of sectors. Figure B.4 shows a plate divided into four sectors.
3. Aseptically get growth from the source culture on the inoculating loop.
4. Lift the lid of the sterile agar plate and use it as a shield to prevent airborne contamination.
5. Touch the agar surface toward the periphery of the sector (Fig. B.4).
6. Remove the loop and replace the lid.



B

B.4 Spot Inoculation of a Plate ♦ Each of four sectors is spot inoculated with a different organism by touching the loop to the surface and making a mark about 1 cm in length. Generally, spot inoculations are done toward the edge (rather than the crowded middle) to prevent overlapping growth and/or test results.

7. Sterilize your loop as before. It is especially important to flame it from base to tip now because the loop has lots of bacteria on it.
8. Incubate the plate in an inverted position for the assigned time at the appropriate temperature.

APPENDIX C

Transfer from a Broth Culture Using a Glass Pipette

Glass pipettes are used to transfer a known volume of liquid diluent, media, or culture. Originally pipettes were filled by sucking on them like a drinking straw, but mouth pipetting is dangerous and has been replaced by mechanical pipettors. Three examples are shown in Figure C.1, each with its own method of operation. Your instructor will show you how to properly use the type of pipettor available in your lab.

To use a pipette correctly, you must be able to correctly read the calibration. Examine Figure C.2. The numbers at the top indicate the pipette's *total volume* and its *smallest calibrated increments*. This is a 10.0 mL pipette divided into 0.1 mL increments.

When reading volumes, use the base of the meniscus (Fig. C.3). The volume in the center pipette is read as exactly 3.0 mL because the meniscus is resting on the line. The left pipette is read as 2.9 mL and the right pipette is read as 3.1 mL (0 is always at the top of the pipette). Although the difference in volume between these three pipettes may seem negligible (1 part in 70[1] is about a 1.5% error), it may be enough to introduce significant error into your work.

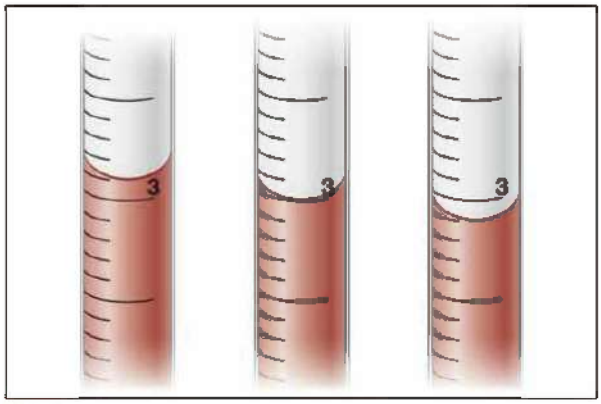
Two pipette styles are used in microbiology (Fig. C.4): the serological pipette and the Mohr pipette. A serological pipette is calibrated to deliver (TD) its volume by completely draining it and blowing out the last drop. The tip of a Mohr pipette is not graduated, so fluid flow must be stopped at a calibration line. Stopping the fluid beyond the last line on a Mohr pipette results in an unknown volume being dispensed. In either case, volumes are read at the bottom of the fluid meniscus.

[1] This number presumes the pipette shown has a total volume of 10 mL. If the meniscus is on the 3, the pipette will dispense 7 mL. If emptied completely, an error of 0.1 mL above or below the 3 would represent 0.1 mL in 7 mL, or 1 part in 70, a 1.4% error that is avoidable.

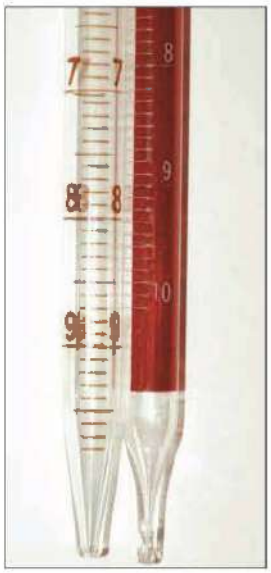
Important: If pipetting a bacterial culture, work over a disinfectant-soaked towel in case any culture drips from the pipette before disposing of it in the autoclave container. This is especially important if transferring a BSL-2 organism. Clean up any spills with additional disinfectant. Also, it is recommended that you use a Mohr pipette to transfer BSL-2 organisms. These are not "blown-out," so aerosol production is reduced.

C.1 Mechanical Pipettors ◆ Three examples of mechanical pipettors are shown here, each with its own method of operation. Your instructor will show you how to properly use the style of pipettor available in your lab. From left to right: A pipette filler/dispenser, a pipette bulb, and a plastic pump.

C.2 Pipette Calibration ◆ Prior to using a pipette, read its calibration. The numbers indicate the pipette's total volume and its smallest calibrated increments. This is a 10.0 mL pipette divided into 0.1 mL increments.



C.3 Read the Base of the Meniscus ♦ When reading volumes, use the base of the meniscus. The volume in the center pipette is read at exactly 3.0 mL because the meniscus is resting on the line. The left pipette is read as 2.9 mL and the right pipette is read as 3.1 mL (0 is always at the top of the pipette).

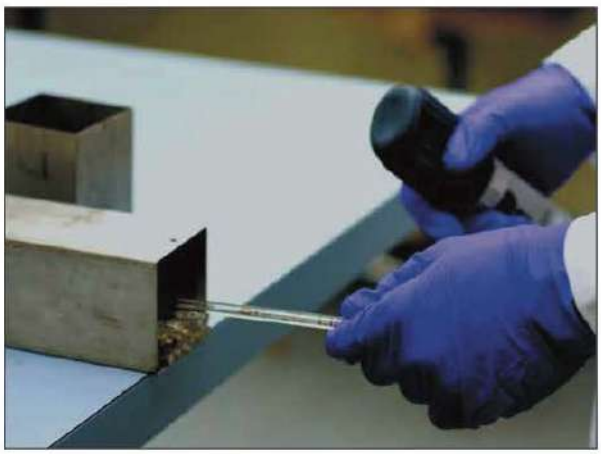


C.4 Two Types of Pipettes ♦ Two pipette styles—the serological pipette (left) and the Mohr pipette (right)—are used in microbiology. A serological pipette is calibrated to deliver (TD) its volume by completely draining it and blowing out the last drop. The tip of a Mohr pipette is not graduated, so fluid flow must be stopped at a calibration line. Stopping the fluid beyond the last line on a Mohr pipette results in an unknown volume being dispensed.

Following are the instructions for using a glass pipette. Be sure to appropriately label the destination tube, bottle, or plate before you start. **Use BSL-2 precautions if appropriate.**

Filling a Glass Pipette

1. Bacteria should be suspended in the broth with a vortex mixer (Fig. 1.17) or by agitating with your fingers (Fig. 1.18). Be careful not to splash the broth into the cap or lose control of the tube.
2. Pipettes are sterilized in metal canisters, individually in sleeves, or as multiples in packages (if disposable). They typically are stored in groups of a single size (Fig. C.5). *Be sure you know what volume your pipette will deliver.* Set the canister at the table edge and remove its lid. (Canisters should not be stored in an upright position, because they may fall over and break the pipettes or become contaminated.) If using pipettes in a package, open the end *opposite the tips.* Grasp *one pipette only* and remove it.
3. Carefully insert the pipette into the mechanical pipettor (Fig. C.6). It is best to grasp the pipette near the end with your fingertips. This gives you more control and reduces the chance that you will break the pipette and cut your hand. *Do not touch* any part of the pipette that will contact the specimen or the medium or you will risk introducing a contaminant. Also, do not lay the pipette on the tabletop while you continue.



C.5 Getting the Sterile Pipette ♦ Pipettes of the same size are autoclaved in canisters, which then are opened and placed flat on the table. Pipettes are removed as needed.

C.6 Assembling the Pipette ♦ Carefully insert the pipette into a mechanical pipettor. Notice that the pipette is held near the end with the fingertips. For safety, the hand is out of the way in case the pipette breaks.

4. While keeping your pipette hand still, bring the culture tube toward it. Use your little finger to remove and hold its cap.
5. Flame the open end of the tube by passing it through a Bunsen burner flame two or three times.
6. Hold the tube at an angle to prevent contamination from above.
7. Insert the pipette and withdraw the appropriate volume (Fig. C.7). Bring the pipette to a vertical position briefly to read the meniscus accurately. (*Remember*: The volumes in the pipette are correct only if the meniscus of the fluid inside is resting *on* the line, not below it.) Then carefully remove the pipette from the tube.
8. Flame the tube lip as before. Keep your pipette hand still.
9. Keeping the pipette hand still (remember, it may contain fluid with microbes in it), move the tube to replace its cap.
10. What you do next depends on the medium to which you are transferring the liquid. Please continue with the appropriate inoculation section.

C.7 Filling the Pipette ◆ Carefully draw the fluid into the pipette. Briefly bring it to a vertical position and read the volume. Notice how the tube's cap is being held by the pinky finger of the pipettor hand.

Inoculation of Broth Tubes with a Pipette

Pipettes are often used to inoculate a known volume of culture into a tube or bottle containing a known volume of diluent during serial dilutions.

1. While keeping the pipette hand still, bring the dilution tube/bottle tube toward it. Use your little finger to remove and hold its cap.
2. Flame the lip of the tube/bottle by quickly passing it through the Bunsen burner flame two or three times. Keep your pipette hand still.
3. Hold the open tube/bottle on an angle to minimize airborne contamination. Keep your pipette hand still.
4. Insert the pipette tip and gently dispense the correct volume of inoculum. Aerosols can be produced if you dispense too quickly.
5. Withdraw the tube/bottle from over the pipette. Before completely removing it, touch the pipette tip to the glass to remove any excess broth.
6. Completely remove the pipette, but avoid waving it around. This can create aerosols.
7. Flame the tube/bottle lip as before. Keep your pipette hand still.
8. Keeping the pipette hand still, move the tube/bottle to replace its cap.
9. The pipette is contaminated with microbes and must be disposed of correctly. Each lab has its own specific procedures, and your instructor will advise you what to do. Glass pipettes typically are placed in a pipette disposal container containing a small amount of disinfectant until they are autoclaved and reused (Fig. C.8). Disposable pipettes must be placed in an appropriate biohazard container. In

C.8 Dispose of the Pipette ◆ The pipette may be contaminated with microbes and must be disposed of correctly. Each lab has its own specific procedures, and your instructor will advise you what to do. Shown here is a canister used for glass pipettes. Disposable pipettes must be placed in an appropriate biohazard container. In either case, be careful when removing the pipette from the mechanical pipettor. There is danger of culture dripping from the pipette or of breaking the glass or plastic.

either case, be careful when removing the pipette from the mechanical pipettor. There is danger of culture dripping from the pipette or of breaking the glass or plastic.

Inoculation of Agar Plates with a Pipette

Pipettes also can be used to dispense a known volume of inoculum to an agar plate.

1. Lift the lid of the plate and use it as a shield to protect it from airborne contamination. It also will help in containing any aerosols you inadvertently produce.
2. Hold the pipette over the agar and dispense the correct volume (often 0.1 mL) onto the center of the agar surface (Fig. C.9). From this point, the remainder of steps should be completed within about 15 seconds to prevent the inoculum from soaking into the agar.
3. The pipette is contaminated with microbes and must be disposed of correctly. Each lab has its own specific procedures, and your instructor will advise you what to do. Glass pipettes typically are placed in a pipette disposal container containing a small amount of disinfectant until they are autoclaved and reused (Fig. C.8). Disposable pipettes must be placed in an appropriate biohazard container. In either case, be careful when removing the pipette from the mechanical pipettor. There is danger of culture dripping from the pipette or of breaking the glass or plastic.
4. Continue with the spread plate technique (Exercise 1-5).

C.9 Dispensing to an Agar Plate ◆ Open the lid and dispense the inoculum onto the surface of the agar near the middle. Notice how the plate's lid is held to prevent aerial contamination and to contain aerosols. The plate may be placed on a disinfectant-soaked towel in case the pipette drips (not shown).

APPENDIX D

Transfer from a Broth Culture Using a Digital Pipette

Modern molecular biology procedures often involve transferring extremely small volumes of liquid with great precision and accuracy. This has led to the development of digital pipettors (Fig. D.1) that can be set to dispense microliter volumes through milliliter volumes (recall that 1 mL equals 1,000 µl). Common digital pipettors are calibrated to dispense volumes of 1 to 10 µl, 10 to 100 µl, 100 to 1,000 µl, or 1,000 to 5,000 µL.

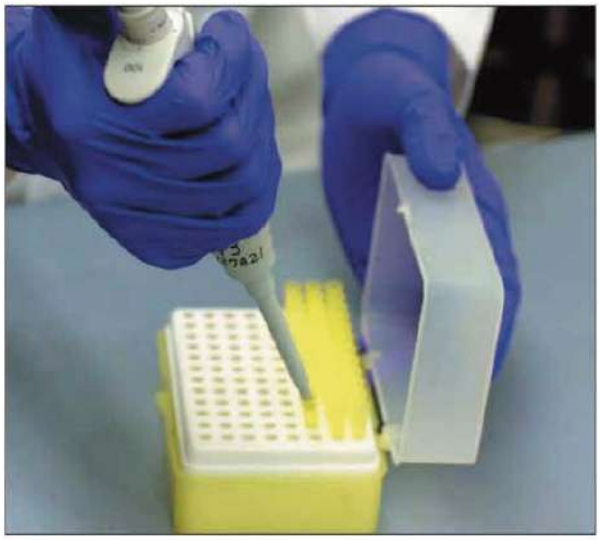
Filling a Digital Pipettor

Many manufacturers make digital pipettors, but they all work basically the same way. The following instructions are for Eppendorf Series 2100 models. **Use BSL-2 precautions, if appropriate.** Before you begin, label the destination tube or plates.

1. Growth may be suspended in the broth with a vortex mixer (Fig. 1.17) or by agitating with your fingers (Fig. 1.18). Be careful not to splash the broth into the cap or lose control of the tube.
2. Determine which digital pipettor should be used to dispense the desired volume.
3. Turn the setting ring to set the desired volume (Fig. D.2). If you turn the dial past the volume range of the pipettor, you will damage it.
4. Hold the digital pipettor in your dominant hand.
5. Open the rack of appropriate pipette tips for your pipettor (these are often color-coded and match the pipettor) and push the pipettor into a sterile tip (Fig. D.3). Close the rack. Never touch the pipette

D.2 Setting the Volume ◆ Rotate the adjustment knob to set the volume on a digital pipettor. This pipettor has been set at 90.0 µl (see inset—the horizontal line between the third and fourth numerals is a decimal point). Never rotate the adjustment knob beyond the volume limits of the pipettor.

D.1 Digital Pipettor ◆ This is a digital pipettor to be used for dispensing volumes between 10 µl and 100 µl. Never set the pipettor to volumes above or below the intended volumes because damage may occur. Also, always use a digital pipettor with the appropriate tip. As shown here, tips and pipettors are often color-coded. If the tips have been sterilized, be sure to close the lid when not picking up a tip.



D **D.3 Digital Pipettor Tips** ◆ Digital pipettors must be fitted with a sterile tip of appropriate size (often color-coded to match the pipettor). Open the case and press the pipettor into a tip, then close the case to maintain sterility. Do not touch the pipettor tip.

tip with your hands or leave the rack open. *Never use a digital pipettor without a tip.*

6 Remove the cap from the culture tube with the little finger of your dominant hand, and flame the tube.

7 Press down the control button with your thumb to the first stop. This is the measuring stroke.

8 Insert the tip into the broth approximately 3 mm while holding it vertically (Fig. D.4).

9 Slowly release pressure with your thumb to draw fluid into the tip. Be careful not to pull any air into the tip.

10 Remove the pipettor and hold it still as you flame the tube as before.

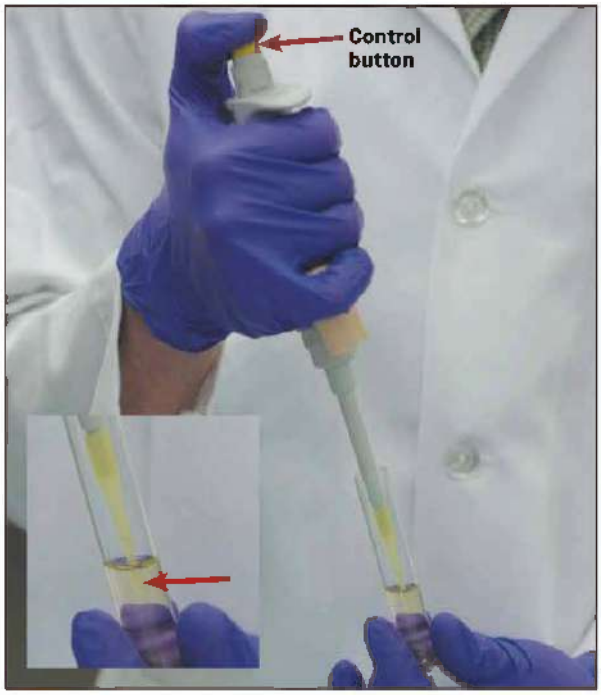
11 Keeping the pipettor hand still, move the tube to replace its cap.

12 What you do next depends on the medium to which you are transferring the growth. Please continue with the appropriate inoculation section.

Inoculation of Broth Tubes with a Digital Pipettor

1 Remove the cap of the sterile medium with the little finger of your pipettor hand, and hold it there. If using a microtube, simply open the cap and continue with step 3.

2 Flame the tube by quickly passing it through the Bunsen burner flame a couple of times. Keep your pipettor hand still.



D.4 Filling the Pipette ◆ Depress the control button with your thumb to the first stop (measuring stroke). Holding the tube and pipettor in a vertical position, insert the tip deep enough into the fluid (about 3 mm, see inset) that the tip won't be exposed as the liquid level drops during filling. Slowly release pressure with your thumb to fill the pipettor.

3 Insert the pipette tip into the tube. Hold it at an angle against the inside of the glass (Fig. D.5).

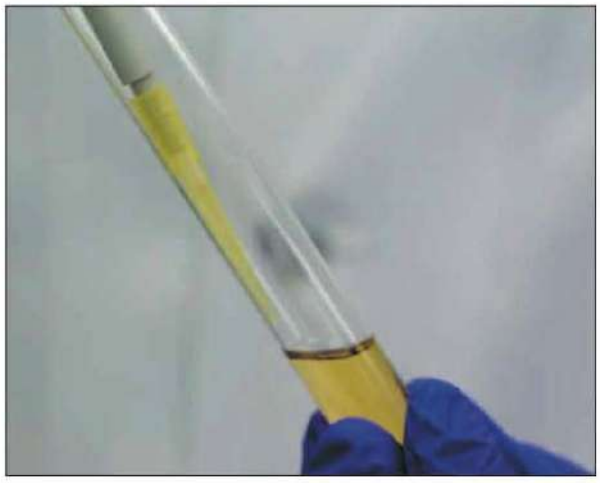
4 Depress the control button slowly with your thumb to the first stop and pause until no more liquid is dispensed. Then continue pressing gently to the second stop to deliver the remaining volume. This is the blow-out stroke, but don't do it so forcefully that you splash liquid.

5 While keeping pressure on the control button, carefully remove the pipettor from the tube by sliding it along the glass. Once it is out of the tube, slowly release pressure on the control button.

6 Keeping your pipette hand still, flame the tube lip as before. If using a microtube, close the cap and continue with step 8.

7 Keeping the pipette hand still, move the tube to replace its cap.

8 The pipettor tip is contaminated with microbes and must be disposed of correctly. Use the ejector button to remove the tip into an appropriate biohazard container (Fig. D.6). Each lab has its own specific procedures, and your instructor will advise you what to do.



D.5 Dispensing to a Liquid ◆ Hold the pipettor at an angle with the tip against the glass. Press the control button to the first stop. When no more fluid comes out, continue pressing to the second stop. Remove the pipettor, then slowly release pressure on the control button, and eject the tip into a biohazard container. Finally, replace the cap on the tube.

D.6 Ejecting the Tip ◆ Using the tip ejector button, eject the contaminated tip into an appropriate biohazard container.

Inoculation of Agar Plates with a Pipettor

1. Lift the lid of the plate and use it as a shield to protect from airborne contamination as well as limiting the spread of any aerosols.
2. Place the pipette tip over the agar surface. Be sure to hold the pipettor in a vertical position (Fig. D.7).
3. Depress the control button slowly with your thumb to the first stop, and pause until no more liquid is dispensed. Then continue gently pressing to the second stop to deliver the appropriate volume. This is the blow-out stroke. From this point, the remaining steps should be completed within about 15 seconds to prevent the inoculum from soaking into the agar.
4. Because the pipettor tip is contaminated with microbes, you must dispose of it correctly. Use the ejector button to remove the tip into an appropriate biohazard container (Fig. D.6). Each lab has its own specific procedures, and your instructor will advise you what to do.
5. Continue with the spread plate technique (Exercise 1-5).

D.7 Dispensing to a Plate ◆ Hold the pipette in a vertical position and press the control button to the first stop. When no more fluid comes out, continue pressing to the second stop. Slowly release pressure on the control button, and then eject the tip into a biohazard container. Notice how the lid is held as a shield to prevent aerial contamination and to contain aerosols.

Modifications for Transferring BSL-2 Microorganisms

Several modifications to the instructions above can be made to minimize aerosol production when using a digital pipettor. They may also be employed for other transfers, but are especially important when transferring BSL-2 organisms. In all cases, transfers should be made over a disinfectant soaked towel in case inoculum leaks from the pipette tip. Put more disinfectant on any spills. Also, keep the culture and destination media as close together as is safe in order to minimize hand movements.

Following are three suggested modifications for transferring BSL-2 organisms with a digital pipettor.

- The pipette tip containing inoculum may be inserted into the broth. Then, gently deliver the inoculum into the broth. Thus, the "blow-out" stroke (pressing to the second stop) occurs within the broth and does not produce aerosols. However, if you use too much force you can cause bubbling of the broth with concurrent aerosol production, so be sure to *gently* dispense the inoculum. Also be sure to keep pressure on the control button as you withdraw the pipette from the broth or you risk changing an intended concentration as you pull back some broth. Use the ejector button to dispose of the tip according to your lab's conventions with a minimum amount of pipettor movement.
- If you are delivering the inoculum to a plate, keep the lid down low and *gently* dispense as far under the lid as possible while still holding the pipettor more-or-less vertically. This will help to contain aerosols. Use the ejector button to dispose of the tip according to your lab's conventions with a minimum amount of pipettor movement.
- Another method involves using the pipettor "in reverse." Instead of pressing to the first stop to fill, filling can be done by pressing to the *second* stop. Then, instead of dispensing by pressing to the second stop, delivery of the inoculum is done by pressing to the *first* stop to avoid the inevitable spraying that occurs when completely emptying the pipette tip. Use the ejector button to dispose of the tip and the remaining inoculum within it according to your lab's conventions with a minimum amount of pipettor movement.

D

Reference

Brinkman Instruments, *Eppendorf Series 2001 Pipette Instruction Manual.* Westbury, New York.

APPENDIX E

The Spectrophotometer

Theory

The spectrophotometer (Fig. E.1) is typically used in quantitative analysis; that is, to determine how much of something is in a liquid sample. In this manual, protocols in Exercises 6-4, 7-2, and 10-1 use spectrophotometry to measure turbidity in liquid for a variety of purposes. Following is a brief introduction to the theory of spectrophotometry and instructions on using the spectrophotometer.

When light strikes a solution, any of several outcomes may occur. The light may be transmitted through it, it may be absorbed, it may be reflected, it may cause fluorescence, or it may be scattered. The spectrophotometer is designed to shine a beam of single wavelength light on a sample and measure % Transmittance (%T) or absorbance of that light. (Under the conditions in the spectrophotometer, reflection, fluorescence, and scatter are negligible.)

When light is shined through an absorbing medium, each fraction of equal thickness absorbs an equal fraction of the light passing through it (Lambert's law). (For example, each 10% portion of the medium absorbs an equal amount.) Further, as long as the light doesn't change the physical or chemical state of the medium, the fraction of light transmitted by a given thickness is independent of the light's intensity. The fraction transmitted then may be expressed as:

$$T = \frac{I}{I_o}$$

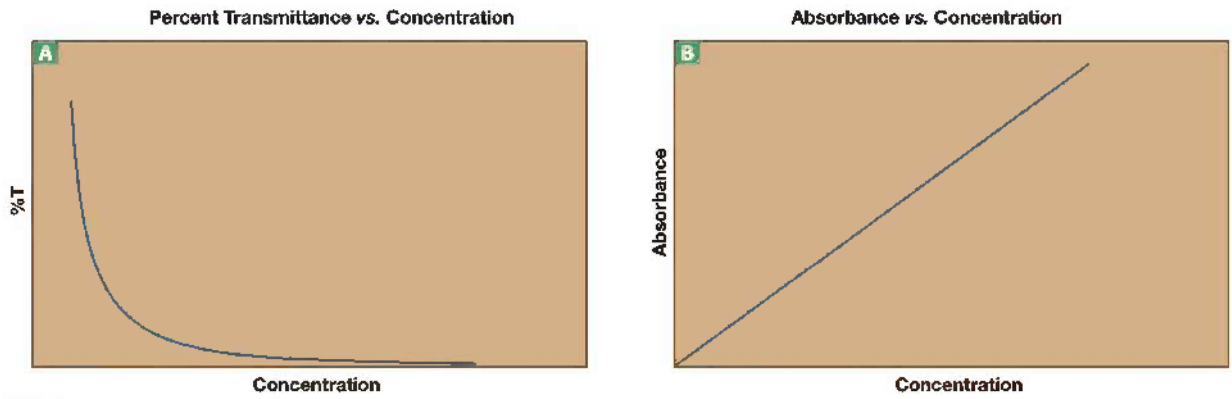
Where I_o is the intensity of incident light and I is the intensity of the transmitted light. The percent transmittance is calculated by multiplying T by 100, as shown.

$$\%T = \frac{I}{I_o} \times 100$$

Plotting concentration of the absorbing medium against %T produces an exponential curve (Fig. E.2A) that is cumbersome to use. Fortunately, concentration of a substance in a solution is directly proportional to the

E.1 The Spectronic 20D+ ◆ This spectrophotometer has a digital display. It is showing an absorbance of 0.364 at a wavelength of 657 nm. The white mode button is used to choose between absorbance and transmittance (note the red light next to absorbance). The black square on the left is the lid to the sample compartment (SC). The knob to its right is used to select wavelength (W). The lever (L) on the lower left positions and removes a filter for use with wavelengths of 600 to 950 nm. The knob on the front left is used to turn the machine on and off, as well as to set 0% Transmittance. The knob on the front right is used to set 100%T with a blank in the sample compartment.

(Photo courtesy of Thermo Spectronic, Rochester, NY)



E.2 Transmission and Absorbance as a Function of Concentration ◆ (A) Transmittance varies exponentially with concentration. This makes using the graph for interpolation difficult, because many points are required to produce the standard curve. (B) Absorbance and concentration are linearly related, making production and interpretation of a standard curve easier.

absorbance (A) of the solution (Beer-Lambert law), provided that the incident light is a single wavelength and that the light doesn't change the physical or chemical state of the medium. Figure E.2B illustrates the relationship between concentration and absorbance.

E The mathematical relationship between absorbance and percent transmittance is as follows:

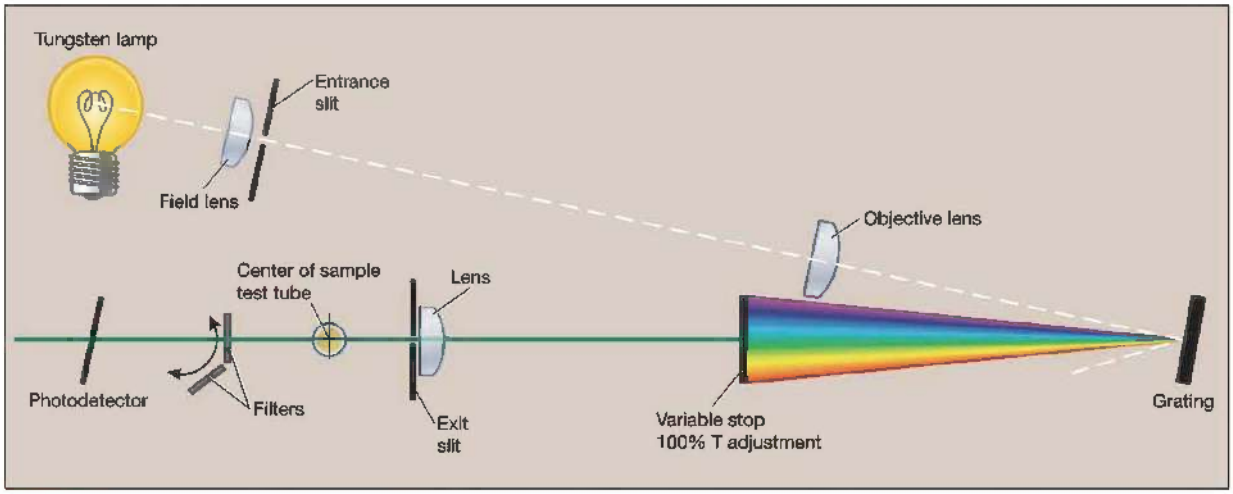
$$A = \log\frac{1}{T} = \log\frac{I_o}{I} = \log I_o - \log I$$

This equation may be modified to:

$$A = 2 - \log\%T$$

because the spectrophotometer is calibrated such that the value of I_o is 100% (2 is the log of 100). Most spectrophotometers allow reading of either %T or A rather than leaving the calculation up to the user.

A simplified schematic of how a spectrophotometer works is shown in Figure E.3. The beam of white light is broken up into its component colors by either a prism or a diffraction grating. The particular wavelength to be used is selected and allowed to pass through the exit slit. It then is directed through the sample contained in a glass or plastic cuvette with a 1 cm light path. The emerging transmitted light strikes a photodetector,



E.3 A Schematic of the Spectrophotometer ◆ Light from the source lamp is focused on the diffraction grating by the field and objective lenses. The grating is responsible for breaking up the white light into its component wavelengths (colors). The desired wavelength is chosen by rotating the grating and aiming that wavelength through the exit slit. This incident light (I_o) then passes through the sample (with a light path of 1 cm) and into the phototube as transmitted light (I). The phototube then converts the transmitted light energy into an electrical signal that produces the readout on the display. Other components include the shutter, which closes when no sample is in place so the machine can be "zeroed," and the filter, which is used with wavelengths of 600 to 950 nm.

(Diagram modified from Spectronic 20D+ literature, courtesy of Thermo Spectronic, Rochester, NY.)

which converts the light energy into an electrical signal proportional to the light intensity. This electrical signal is amplified and displayed by the spectrophotometer as either percent transmittance or absorbance.

In quantitative analysis, absorbance of the compound or particle being examined must be differentiated from absorbance because of the solvent in which it is dissolved and the cuvette itself. This is done by calibrating the spectrophotometer with an identical blank cuvette containing the solvent but lacking the compound or particle being measured.

Instructions for Using the Spectronic D20+

1. Turn on the spectrophotometer and allow it to warm up for 15 minutes.
2. Choose the wavelength to be used by turning the wavelength control knob on the top of the machine.
3. Make sure the filter is set for the correct range of wavelength to be used.[1]
4. Set the machine to read % Transmittance by pressing the mode button until the display indicates Transmittance.[2]
5. Prepare a "blank" cuvette identical to the experimental cuvette minus the particles you are measuring. (For instance, sterile medium if you are checking absorbance of bacterial cells.)
6. Use care to handle the cuvettes by the top only, because fingerprints influence results. Use tissue to remove fingerprints.
7. Use the power/zero control knob to set the machine to 0%T with the sample compartment empty and the lid closed.
8. If measuring %T, go to step 9. If measuring A, go to step 10.
9. Be sure the blank is mixed uniformly, but do not introduce bubbles. Insert the blank into the sample compartment and close the cover. Use the right control knob (100% T/OA) to set 100%T. Continue with step 11.
10. Select absorbance mode. Be sure the blank is mixed uniformly, but do not introduce bubbles. Then insert the blank into the sample compartment and close the cover. Use the light control knob to set zero A. Continue with step 11.
11. Remove the blank.
12. Be sure the experimental cuvette is mixed uniformly, but do not introduce bubbles. Insert it into the sample compartment and close the lid. Read the appropriate display and record the value.
13. It is advisable to calibrate the spectrophotometer prior to each use if there is an interval of more than a few minutes between readings.
14. When finished with all readings, turn off the spectrophotometer.
15. *Note:* Your instructor must be notified immediately if you spill any culture into the spectrophotometer. You will be instructed on how to decontaminate the spill.

[1] On the older Spectronic 20 machines, a red filter will have to be manually inserted if wavelengths longer than 600 nm are used.

[2] On the Spectronic 20, the display has both %T and A scales.

References

Abramoff, Peter and Robert G. Thompson. Appendix A and Appendix B in *Laboratory Outlines in Biology—III*. San Francisco: W. H. Freeman and Co., 1982.

Thermo Spectronic. *Theory of UV-Visible Spectrophotometry*. Rochester, NY: Thermo Spectronic. Available online. URL: http://www.thermo.com/eThermo/CDA/KnowledgeBase/ Product_Knowledge_Base_Detail/0,1292,12100-119-10023,00.html.

APPENDIX F

Alternative Procedures for Section 6

Alternative Procedure for Exercise 6-1, Standard Plate Count (Viable Count)[1]

This protocol is modified to allow use of digital pipettors. Different and smaller volumes are used, but the dilutions are exactly the same as in the original Exercise 6-1.

✓ Materials

Per Student Group

- ☐ Micropipettes (10–100 µL and 100–1,000 µL) with sterile tips
- ☐ Five sterile microtubes
- ☐ 50 mL flask of sterile water
- ☐ Eight nutrient agar plates
- ☐ Jar (and its lid) containing ethanol and a bent glass rod
- ☐ Hand tally counter
- ☐ Colony counter
- ☐ 24-hour broth culture of *Escherichia coli* (this culture will have between 2×10^7 and 2×10^{10} CFU/mL)

Procedure

Refer to the procedural diagram in Figure F.1 as needed.

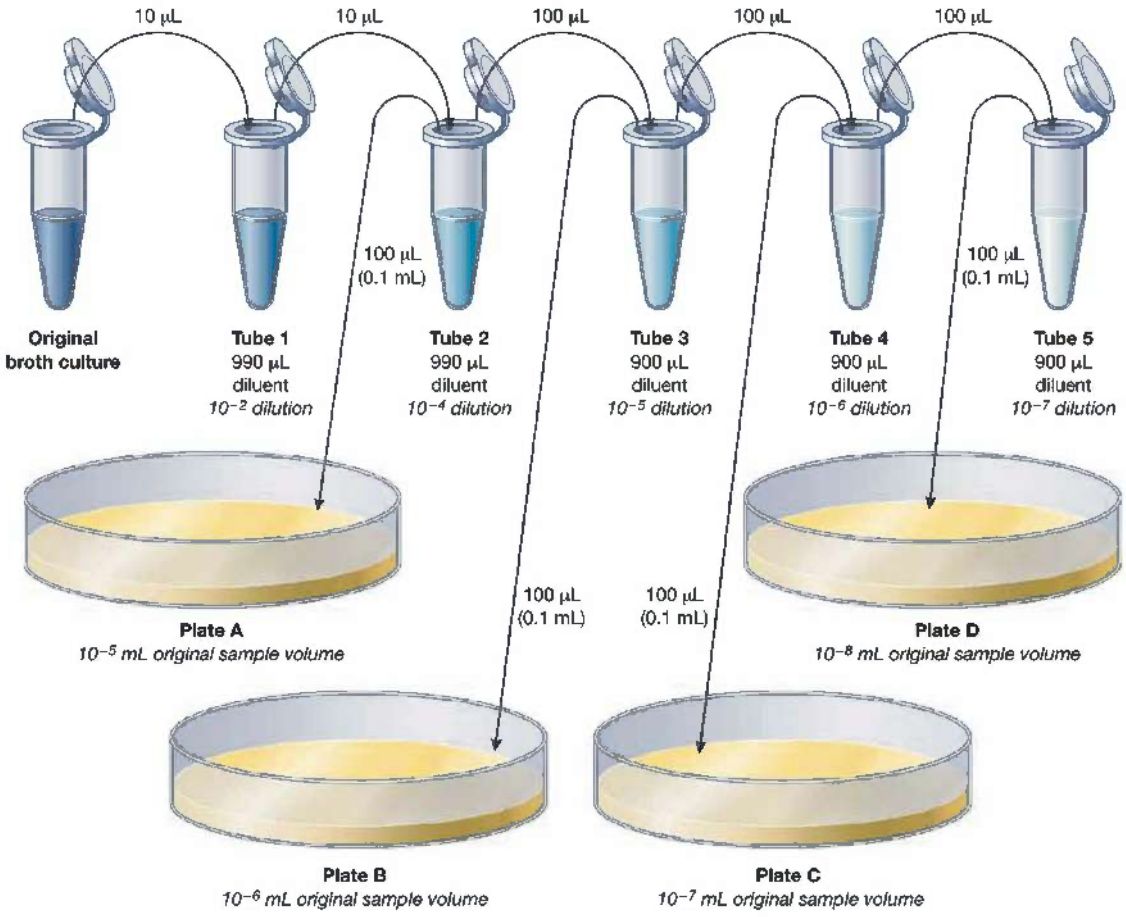
Lab One

1. Obtain eight plates, organize them into four pairs, and label them A_1, A_2, B_1, B_2, etc.
2. Obtain five microtubes, and label them 1–5. These are your dilution tubes; make sure they remain covered until needed.
3. Aseptically add 990 µL sterile water to dilution tubes 1 and 2. Close the caps when finished. Aseptically add 900 µL sterile water to dilution tubes 3, 4, and 5. Close the caps when finished.
4. Mix the broth culture, and aseptically transfer 10 µL to dilution tube 1; mix well. This is a 10^{-2} dilution.
5. Aseptically transfer 10 µL from dilution tube 1 to dilution tube 2; mix well. This is a 10^{-4} dilution.
6. Aseptically transfer 100 µL from dilution tube 2 to dilution tube 3; mix well. This is a 10^{-5} dilution.
7. Aseptically transfer 100 µL from dilution tube 3 to dilution tube 4; mix well. This is a 10^{-6} dilution.
8. Aseptically transfer 100 µL from dilution tube 4 to dilution tube 5; mix well. This is a 10^{-7} dilution.
9. After mixing, aseptically transfer 100 µL from dilution tube 2 to plate A_1. Using the spread plate technique (Exercise 1-5), disperse the diluent evenly over the entire surface of the agar. Repeat the procedure with plate A_2, and label both plates "10^{-5} mL original sample" or simply "10^{-5}."
10. Following the same procedure, transfer 100 µL volumes from dilution tubes 3, 4, and 5 to plates B, C, and D, respectively. Label the plates with their appropriate original sample volumes.
11. Invert the plates, and incubate at 35 ± 2°C for 24 to 48 hours.

Lab Two

Continue with Lab Two instructions in the main exercise on page 472.

[1] Other, more sophisticated counting methods are available. One uses an electronic pen that, when touched to the plastic Petri dish below a colony, records a tally. There are also software systems that capture an image of the plate and then colonies are counted by the computer.



F

F.1 Serial Dilution Procedural Diagram (Measuring Volumes with a Digital Pipette) ◆ This is an illustration of the dilution scheme outlined in the Procedure. The dilution assigned to the dilution tubes and their contents indicate the proportion of original sample present in the tube. Be aware that the original cell density will be expressed in CFU/mL and the easiest way to do that is to have the original sample volumes on the plates also expressed in milliliters. However, the volume of diluted inoculum going to the plates is in microliters (100 µL). Without showing it, we converted the 100 µL to 0.1 mL to calculate the original sample volumes. (Recall that original sample volume on the plate = volume plated x dilution factor.) Making this simple conversion at this point prevents a more complicated conversion later. It is convenient to measure in microliters, but try to think in milliliters. For more information, see Figure 6.1.

Alternative Procedure for Exercise 6-5, Plaque Assay of Virus Titre

This protocol is modified to allow use of digital pipettors. Different and smaller volumes are used, but the dilutions are exactly the same as in the original Exercise 6-5.

✓ Materials

Per Class

- ☐ 50°C hot-water bath containing tubes of liquid soft agar (7 tubes per group)

Per Student Group

- ☐ Micropipettes (10–100 µl and 100–1,000 µl) with sterile tips
- ☐ 14 sterile capped microtubes (1.5 mL or larger) in a microtube holder (rack)
- ☐ Seven tryptic soy agar or nutrient agar plates
- ☐ Seven tubes containing 2.5 mL soft agar
- ☐ 10 mL of sterile normal saline in a small flask
- ☐ Seven sterile transfer pipettes
- ☐ T4 coliphage
- ☐ 24-hour broth culture of *Escherichia coli B* (T-series phage host)

Procedure

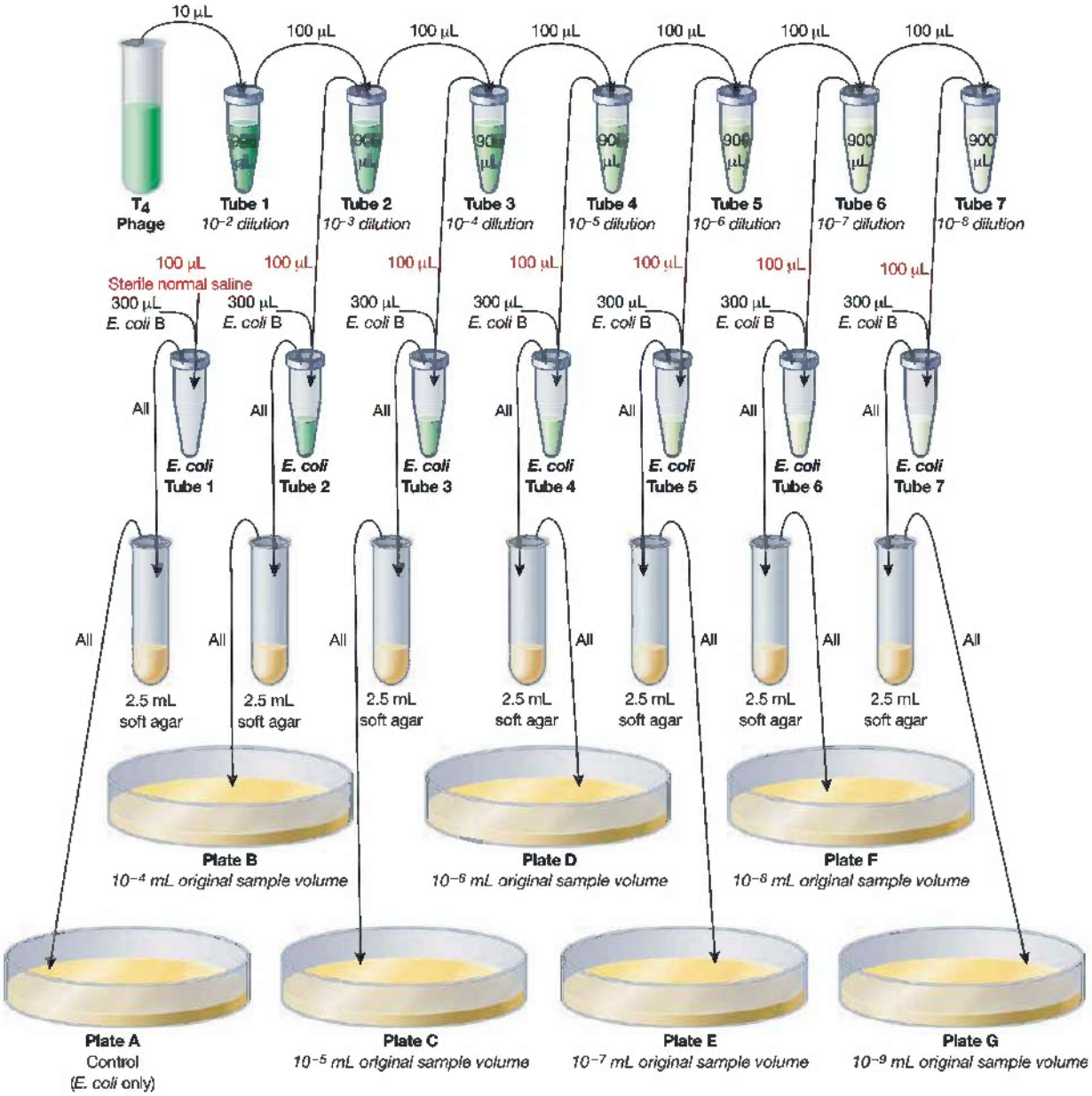
Refer to the procedural diagram in Figure F.2 as needed.

Lab One

1. Obtain all materials except for the soft agar tubes. To keep the agar tubes liquefied, leave them in the water bath and take them out one at a time as needed.
2. Label seven microtubes 1 through 7. Label the other seven microtubes *E. coli* 1–7. Place all tubes in a microtube holder, pairing like-numbered tubes.
3. Label the TSA (or nutrient agar) plates A through G. Place them in the 35°C incubator to warm them. Take them out one at a time as needed. This will keep the soft agar (added at step 14) from solidifying too quickly and result in a smoother agar surface.
4. Aseptically transfer 990 µL sterile normal saline to dilution tube 1.
5. Aseptically transfer 900 µL sterile normal saline to dilution tubes 2–7.
6. Mix the *E. coli* culture and aseptically transfer 300 µL into each of the *E. coli* microtubes.
7. Mix the T4 suspension and aseptically transfer 10 µL to dilution tube 1. Mix well. This is a 10^{-2} dilution.
8. Aseptically transfer 100 µl from dilution tube 1 to dilution tube 2. Mix well. This is a 10^{-3} dilution.
9. Aseptically transfer 100 µl from dilution tube 2 to dilution tube 3. Mix well. This is a 10^{-4} dilution.
10. Continue in this manner through dilution tube 7. The dilution in tube 7 should be 10^{-8}.
11. Aseptically transfer 100 µl sterile normal saline to *E. coli* tube 1. This will be mixed with 2.5 mL soft agar and used to inoculate a control plate.
12. Aseptically transfer 100 µl from dilution tube 2 to its companion *E. coli* tube. Repeat this procedure with the remaining five tubes.
13. This is the beginning of the preadsorption period. Let all seven tubes stand undisturbed for 10 ± 5 minutes.
14. Remove one soft agar tube from the water bath and add the entire contents of *E. coli* tube 1 with a sterile transfer pipette. Mix well with the pipette and immediately transfer it onto plate A. Gently tilt the plate back and forth until the soft agar mixture is spread evenly across the solid medium. Label the plate "Control."
15. Remove a second soft agar tube from the water bath and add the entire contents of *E. coli* tube 2 with a sterile transfer pipette. Mix well with the pipette and immediately transfer it onto plate B. Tilt back and forth to cover the agar and label it 10^{-4} mL (this is the phage sample volume).
16. Repeat this procedure with dilutions 10^{-4} through 10^{-8} and plates C through G. Label the plates with the appropriate original phage sample volumes.
17. Allow the agar to solidify completely.
18. Invert the plates and incubate aerobically at 35 ± 2°C for 24 to 48 hours.

Lab Two

Continue with Lab Two instructions in the main exercise on page 501.



F

F.2 Plaque Assay Procedural Diagram (Measuring Volumes with a Digital Pipette) ◆ This is an illustration of the dilution scheme outlined in the Procedure. The dilutions assigned to the dilution tubes in the first row indicate the proportion of original phage sample present in the tube. Be aware that the original phage density will be expressed in PFU/mL and the easiest way to do that is to have the original sample volumes on the plates also expressed in milliliters. However, the volume of diluted phage going to the plates (via the *E. coli* tube and soft agar tube) is in microliters (100 µL). Without showing it, we converted the 100 µL to 0.1 mL to calculate the original sample volumes. (Recall that original sample volume on the plate = volume plated x dilution factor.) Making this simple conversion at this point prevents a more complicated conversion later. It is convenient to measure in microliters, but try to think in milliliters. For more information, see Figure 6.14.

Alternative Procedure for Exercise 6-6, Thermal Death Time Versus Decimal Reduction Value

This protocol is modified to allow use of digital pipettors. Different and smaller volumes are used, but the dilutions are exactly the same as in the original Exercise 6-6.

✓ Materials

Per Class

- ☐ Stopwatch
- ☐ Thermometers
- ☐ Two flasks, each containing 49.0 mL nutrient broth, maintained at 60°C in a water bath
- ☐ 24-hour broth cultures of:
- ☐ *Escherichia coli*
- ☐ *Staphylococcus epidermidis*

Per Group

- ☐ Seven nutrient agar plates
- ☐ 16 nutrient broth tubes
- ☐ Digital pipettor (100-1,000 µL)
- ☐ Sterile 100 µl micropipette tips
- ☐ Sterile 1,000 µl micropipette tips
- ☐ Container for pipette tip disposal
- ☐ Sterile 1.5 mL or larger microtubes
- ☐ Sterile deionized water in a small flask or beaker
- ☐ Screw-cap jar (with lid) of alcohol and bent glass rod for spreading organisms
- ☐ Bucket of crushed ice (optional)

Procedure

Preliminary Notes

- This procedure requires knowledge of dilutions and calculations, explained fully in Exercise 6-1, Standard Plate Count. If you have not completed a standard plate count, or you have any doubt about your ability to complete this exercise, we suggest that you read Exercise 6-1 carefully and do some or all of the practice problems.
- This procedure uses the spread plate technique described in Exercise 1-5. Refer to it as needed.
- The Plate Subgroups will need at least two or three students for labeling microtubes and plates, performing the serial dilution, and spreading plates.
- The Broth Subgroups will need one or two students to label and inoculate the 15 nutrient broths, keep track of time, and mix the broth prior to all transfers.
- The dilution/plating process can be slow for novices, so we recommend keeping dilution broths on ice until they can be plated to minimize bacterial reproduction.

Lab One: Group 1 (t_0)

Plate Subgroup

1. Obtain all plating materials. Place seven microtubes in a rack and label them 1 through 7. Add 900 µL of sterile water to microtubes 2 through 7, and cap them until needed.
2. Label seven nutrient agar plates 1 through 7.
3. When your instructor adds 1.0 mL of the *E. coli* culture to the heated flask, he/she will start the stopwatch. This is t_0.
   - Immediately swirl the flask to mix its contents. Remove 1,000 µL of broth from the flask and transfer it to tube 1. If possible, do this step without removing the flask from the water bath. Continue with the serial dilution below.
   - Without changing the pipette tip, transfer 100 µL from tube 1 to tube 2; mix. Properly discard the pipette tip according to your lab's protocol. Use a clean pipette tip for each transfer from here forward.
   - Transfer 100 µL from tube 2 to tube 3; mix.
   - Transfer 100 µL from tube 3 to tube 4; mix.
   - Transfer 100 µL from tube 4 to tube 5; mix.
   - Transfer 100 µL from tube 5 to tube 6; mix.
   - Transfer 100 µL from tube 6 to tube 7; mix.
   - Transfer 100 µL from tube 1 to plate 1 and spread according to spread plate procedure.
   - Transfer 100 µL from tube 2 to plate 2 and spread.
   - Transfer 100 µL from tube 3 to plate 3 and spread.
   - Transfer 100 µL from tube 4 to plate 4 and spread.
   - Transfer 100 µL from tube 5 to plate 5 and spread.
   - Transfer 100 µL from tube 6 to plate 6 and spread.
   - Transfer 100 µL from tube 7 to plate 7 and spread.
4. Calculate the volume of original sample transferred to each plate (volume plated x dilution) and enter it in Table 1 on the data sheet, page 511. If you did Exercise 6-1, you have calculated original sample volumes before!
5. Tape the plates together, invert, and incubate them at 35 ± 2°C for 48 hours.

Broth Subgroup

1. Obtain all broth transfer materials. Label 15 nutrient broth tubes t_1 through t_{15}. Label the 16th

"Control." Enter these numbers in the appropriate boxes in Table 2 on the data sheet, page 511.

2. When your instructor adds 1.0 mL of the *E. coli* culture to the heated flask, he/she will start the stopwatch. This is t_0. Slightly before 1 minute (t_1), gently swirl the flask to mix the broth. At exactly t_1, transfer 100 µL from the flask to the appropriately labeled nutrient broth tube. Properly discard the pipette tip according to your lab's protocol.
3. Repeat this procedure at t_2 through t_{15}. Be sure to mix the broth before each transfer, and use a clean pipette tip.
4. Incubate all broths at 35 ± 2°C for 48 hours.

Lab One: Group 2 (t_{10})

Plate Subgroup

1. Obtain all plating materials. Place six microtubes in a rack and label them 1 through 6. Add 900 µL of sterile water to microtubes 2 through 6, and cap them until needed.
2. Label seven nutrient agar plates 1 through 7.
3. When your instructor adds 1.0 mL of the *E. coli* culture to the heated flask, he/she will start the stopwatch. This is t_0 and your work won't begin until 10 minutes later (t_{10}).
   - At T_{10}, swirl the flask to mix its contents. After the broth group withdraws their t_{10} sample, remove 1,500 µL with your pipette and transfer it to tube 1. Unless you have 5,000 µL pipettes, this will take two 750 µL withdrawals. Continue with the serial dilution below.
   - Without changing the pipette tip, transfer 100 µL from tube 1 to tube 2; mix. Properly discard the pipette tip according to your lab's protocol. Use a clean pipette tip for each transfer from here forward.
   - Transfer 100 µL from tube 2 to tube 3; mix.
   - Transfer 100 µL from tube 3 to tube 4; mix.
   - Transfer 100 µL from tube 4 to tube 5; mix.
   - Transfer 100 µL from tube 5 to tube 6; mix.
   - Transfer 100 µL from tube 6 to tube 7; mix.
   - Transfer 100 µL from tube 1 to plate 1 and spread according to the spread plate procedure.
   - Transfer 100 µL from tube 1 to plate 2 and spread.
   - Transfer 100 µL from tube 2 to plate 3 and spread.
   - Transfer 100 µL from tube 3 to plate 4 and spread.
   - Transfer 100 µL from tube 4 to plate 5 and spread.
   - Transfer 100 µL from tube 5 to plate 6 and spread.
   - Transfer 100 µL from tube 6 to plate 7 and spread.
4. Calculate the volume of original sample transferred to each plate (volume plated x dilution) and enter it in Table 1 on the data sheet, page 511. If you did Exercise 6-1, you have calculated original sample volumes before!
5. Tape the plates together, invert, and incubate them at 35 ± 2°C for 48 hours.

Broth Subgroup

1. Obtain all broth transfer materials. Label 15 nutrient broth tubes t_{16} through t_{30}. Label the 16th "Control." Enter these numbers in the appropriate boxes in Table 2 on the data sheet, page 511.
2. When your instructor adds 1.0 mL of the *E. coli* culture to the heated flask, he/she will start the stopwatch. This is t_0, and your work won't begin until about 15 minutes later. Slightly before t_{16} (16 minutes after the start), gently swirl the broth flask and at exactly t_{16}, transfer 100 µl from the flask to the appropriately labeled Nutrient Broth tube. Properly discard the pipette tip according to your lab's protocol.
3. Repeat this procedure at t_{17} through t_{30}. Be sure to mix the broth before each transfer, and use a clean pipette tip.
4. Incubate all broths at 35 ± 2°C for 48 hours.

Lab One: Group 3 (t_0)

Plate Subgroup

Using the *S. epidermidis* culture, follow the instructions for the Group 1 Plate Subgroup.

Broth Subgroup

Using the *S. epidermidis* culture, follow the instructions for the Group 1 Broth Subgroup.

Lab One: Group 4 (t_{10})

Plate Subgroup

Using the *S. epidermidis* culture, follow the instructions for the Group 2 Plate Subgroup.

Broth Subgroup

Using the *S. epidermidis* culture, follow the instructions for the Group 2 Broth Subgroup

Lab Two: All Groups

All groups should continue with Lab Two instructions in the main exercise on page 510.

APPENDIX G

Agarose Gel Electrophoresis

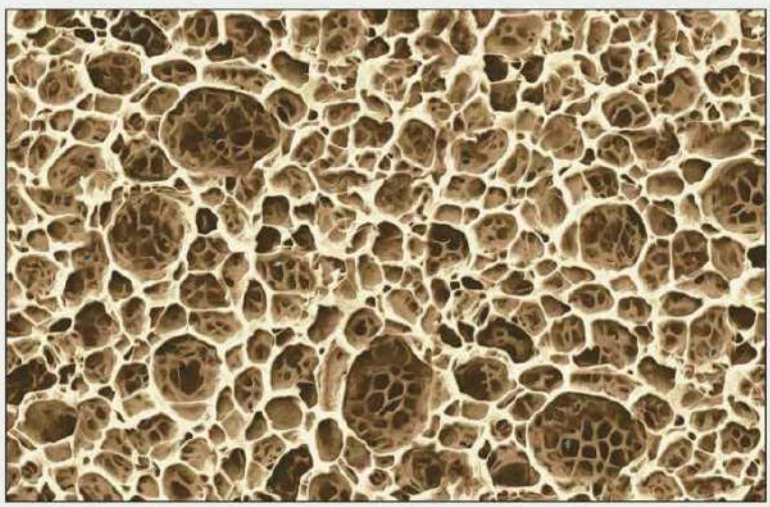
This exercise is designed to supplement Exercise 10-2 Restriction Digest, and Exercise 10-4 Polymerase Chain Reaction. It is divided into three modules: Module A–Gel Preparation, Module B–Loading and Running Samples, and Module C–Gel Staining and Destaining. Approximate times for completion are included to facilitate scheduling of lab periods; however, many variables affect each step so the time necessary will depend on your particular experimental conditions. The whole procedure may be broken up over several days as necessary.

The instructions presented in the three modules are designed to serve as a general guide. The exact procedure and quantities of materials being used will depend on the particular equipment available in your laboratory and time allotted to the experiment. Your instructor will provide you with the necessary details.

Theory

Electrophoresis is a technique in which molecules are separated by size and electrical charge in a gel subjected to an electrical field. Electrophoresis gels are typically prepared from agarose or polyacrylamide, depending on the molecules to be separated. Agarose is typically used for larger DNA molecules and polyacrylamide is used for smaller DNA molecules and proteins. Both substances begin as liquid solutions that will polymerize into solid-appearing but actually porous gels. The microscopic matrix of polymerized strands (Fig. G.1) function as a sieve by retarding the movement of larger molecules while allowing smaller molecules to migrate easily through the spaces between the strands. All else being equal, the smaller the molecule, the more easily it passes through the pores and therefore the farther it travels in the gel. When molecules of similar size are to be separated, higher concentrations of the polymerizing agent are used to create smaller pores in the matrix, which are able to resolve small size differences by providing greater resistance to migration.

In preparation for the procedure, the gel is cast as a thin slab (a few millimeters) containing tiny wells at one end. The gel is placed in a buffered solution to maintain proper electrolyte balance. Samples to be examined are loaded into the different wells and electrodes are attached



G.1 Artist's Rendition of a Scanning Electron Micrograph of an Agarose Gel

to create an electrical field in the gel. DNA and most proteins are negatively charged so when they are loaded into wells near the negative electrode, they will be repelled by the negative, and attracted to the positive electrode on the opposite end of the gel. While moving, because of electrical repulsion and attraction, the molecules pass through the pores of the gel matrix at different rates, depending on their size. They also migrate in a straight line, called a lane, from their well toward the positive electrode. This means fragments from one well do not mix with neighboring fragments, so when the gel is complete each sample may be observed separately in the lane beneath its well.

If an appropriate gel concentration is used, fragment separation can be very discriminating. DNA segment length and protein size can be accurately determined by running samples through the gel alongside a DNA ladder, a collection of DNA fragments of known lengths in kilobase pairs (kb); or protein molecular weight markers, a collection of proteins of known molecular mass in kilodaltons (kDa).

The gel matrix is equally porous, which means that if it is run too long all fragments in a sample can, theoretically, travel out the other end of the gel into the electrolyte buffer. Because protein and DNA are colorless, a visual marker is necessary to observe the progress of molecular movement in the lanes during the run. Tracking dyes that migrate at predictable rates compared to the size of the proteins or DNA fragments are usually added to the samples to see the progress of the run. These dyes do not stain the DNA or proteins, however, since doing this would alter the movement of these molecules in the gel and make it more difficult to accurately predict the size.

At the end of the run, the gel is stained to show the location of the separated molecules as bands in each lane, in the same rectangular shape as the well where they began. Coomassie blue is commonly used for protein, whereas ethidium bromide, a fluorescent dye, or methylene blue can be used for nucleic acids. If the separated molecules have been radioactively labeled, the gel can be placed on X-ray film and the radioactive bands expose the film to produce an autoradiograph.

Application

Gel electrophoresis is a means of isolating proteins or fragments of DNA for a variety of purposes. Unique band patterns can be used for taxonomic or identification purposes. Once isolated, the DNA or proteins can be removed from the gel and used in biochemical studies. Other techniques, such as DNA fingerprinting and Southern, Western, and Northern blotting, begin with electrophoresis.

Module A—Gel Preparation (~30 minutes to 1 hour)

✓ Materials

Per Gel

(Multiple student groups can share a single gel)

- ☐ Gel casting tray
- ☐ Comb
- ☐ Two rubber dams or tape
- ☐ Powdered agarose
- ☐ Erlenmeyer flask (250 mL)
- ☐ 1X TAE (50–150 mL)
- ☐ Magnetic stirrer hot plate and stir bar
- ☐ Foil
- ☐ 50°C water bath
- ☐ Deionized water
- ☐ Plastic wrap and zipper bag or plastic container (optional)

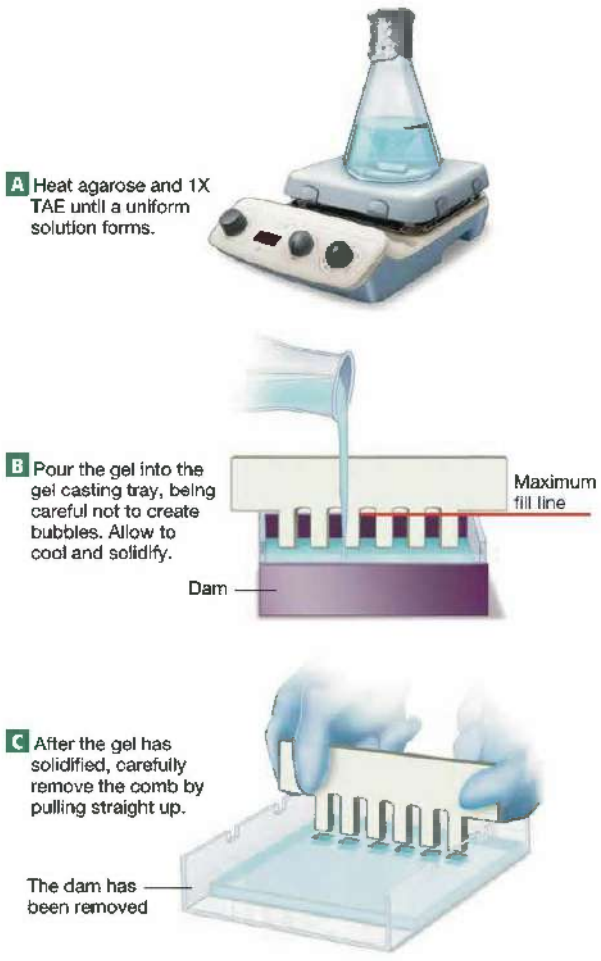
Follow the procedural diagram in Figure G.2.

1. Prepare the gel-casting tray by sealing the ends with rubber dams or tape. Place it on a level surface and insert the comb at one end. Some trays have defined slots for the comb to rest in. If yours does not, make sure it is perfectly parallel to the dam or tape and about a centimeter in from the end.

2. Weigh the amount of agarose necessary to prepare a 0.8%–1.0% gel in 1X TAE buffer for your particular tray size. The higher the agarose percent the better the fragment separation, but the slower the gel will run. The experimental samples you will be loading are 60 µL so if your comb has many narrow wells as opposed to fewer wider wells it is important that your pour the gel deep to allow the well to accommodate maximum volume. Some example recipes are listed in Table G-1.

3. Pour the agarose powder, 1X TAE buffer, and a magnetic stir bar into a 250 mL flask. On the outside of the flask, mark the level of the solution with a marking pen and cover the flask with foil (Fig. G.2A).

4. Heat the solution, stirring constantly, until it boils and completely dissolves the agarose. Be sure to watch the flask and remove it from the heat as needed to prevent a boil over. (***Warning***! Heating in a microwave is not advised because the lack of stirring can lead to super heating, which will result in an explosive boil over when you attempt to pick up the flask.)


A Heat agarose and 1X TAE until a uniform solution forms.
B Pour the gel into the gel casting tray, being careful not to create bubbles. Allow to cool and solidify.
Maximum fill line
Dam
C After the gel has solidified, carefully remove the comb by pulling straight up.
The dam has been removed

G.2 Procedural Diagram for Preparing an Agarose Gel

5 When the agarose is fully dissolved (completely clear with no visible crystals, particles, or swirls), remove the flask from the heat and place it in the 50°C water bath.

6 When the gel has cooled (50°C can be held comfortably in the palm of your hand), check the volume and add water if necessary to bring the level of the solution back up to the mark placed on the outside of the flask in step 3. Stir gently until the mixture is uniformly clear. Either remove the stir bar or hold it in place with another stir bar on the outside of the flask to avoid pouring it into the gel-casting tray.

7 Steadily pour the liquid gel into the casting tray. Try to avoid creating bubbles. If bubbles do appear, use a pipette tip to push them to the end of the gel farthest from the comb. Fill the gel to the top of the dams or tape, or the top of spaces between the wells on the comb (Fig. G.2B), whichever level is reached first. Allow the tray to remain undisturbed until the gel is completely cool and solid.

8 Once solidified, the gel can be used immediately, or stored at 4°C for several days. If storing, leave the comb and dams in place and pour a very thin layer of 1X TAE buffer over the surface to keep the gel moist. Seal the gel securely in plastic wrap and a plastic zipper bag or plastic storage container. When ready to use, remove the comb first by pulling straight up very slowly with steady hands (Fig. G.2C). Then remove the tape or dams. Be careful not to tilt the gel because it is slippery and can easily slide off the casting tray.

TABLE G-1 Example Gel Recipes

Casting Tray Size	Agarose	1X TAE
7 cm × 7 cm	0.4 g (0.8%) to 0.5 g (1%)	50 mL
7 cm × 15 cm	0.8 g (0.8%) to 1.0 g (1%)	100 mL
10.5 cm × 14 cm	1.2 g (0.8%) to 1.5 g (1%)	150 mL

Module B—Loading and Running Samples (~30 minutes to 2 hours)

✓ Materials

Per Gel/Student Group

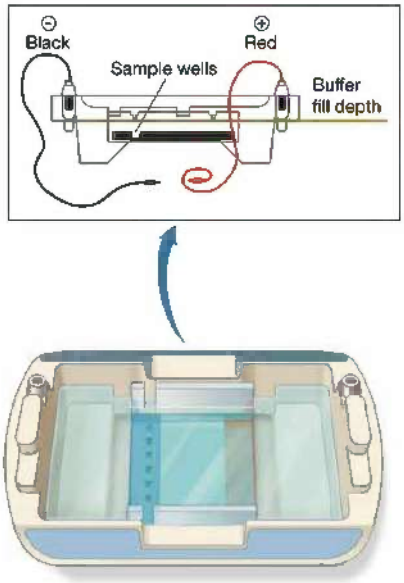
- ☐ Electrophoresis chamber
- ☐ Power supply
- ☐ 1X TAE buffer
- ☐ 6X DNA loading dye
- ☐ Digital pipettor (10-100 µL) and tips

Follow the procedural diagram in Figure G.3.

1 Place the gel in the electrophoresis chamber and completely cover it with 1X TAE buffer up to the line (Fig. G.3A). An alternate name for this type of gel is the "submarine gel," so be certain it is fully submerged.

2 In order to make your samples dense enough to stay in the bottom of the wells that are now submerged in 1X TAE buffer, you must add the appropriate amount of 6X DNA loading dye to bring the final concentration of the sample to 1X DNA loading dye. The loading dye contains glycerol to increase the sample density, and two tracking dyes to allow visualization of molecular movement through the gel as it runs.

3 Draw your first sample up into the pipette tip with no air bubbles. With the micropipettor in your

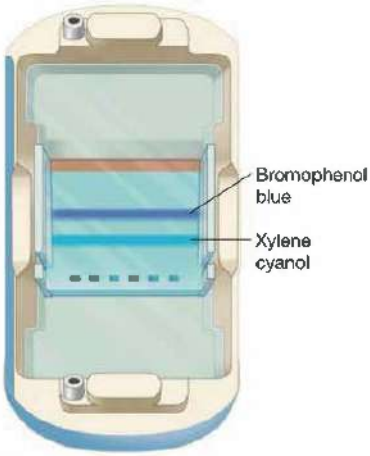


A Place the entire gel casting tray containing the gel into the electrophoresis chamber and cover with buffer. Orient properly with the wells nearest the black terminal.

B Draw up your first sample in the pipette tip with no air bubbles. With the micropipettor in your dominant hand, place your elbow on the table and the pipette tip into the buffer over the well to be loaded. Hover over the well without touching the gel. Use the index finger of your nondominant hand to stabilize the barrel of the micropipettor and slowly eject the sample from the tip, watching it sink down into the base of the well without spilling over the side into the neighboring well.

G

C Plug in and turn on the power supply to the appropriate voltage.



D Run the gel until the bromophenol blue tracking dye is approximately 80% of the way down the gel.

G.3 Procedural Diagram Illustrating Gel Loading and Running ♦

dominant hand, place your elbow on the table and the pipette tip into the buffer over the well to be loaded (Fig. G.3B). Hover over the well without touching the gel. Use the index finger of your non-dominant hand to stabilize the barrel of the micropipettor and slowly eject the sample from the tip, watching it sink down into the base of the well without spilling over the side into a neighboring well. Keep pressure on the plunger while carefully removing the micropipettor so as not to disturb the well contents.

4. Repeat this loading for each sample. Be sure to keep track of which sample was loaded into each well. Change the pipette tip between samples.

5. Carefully attach the electrodes and lid (if your electrophoresis chamber has one) and plug it into the power source (Fig. G.3C). Be sure the negative electrode (black) is at the well end of the gel. Always "Run to Red."

6. Turn on the power source and set it to the appropriate voltage. DNA agarose minigels are typically run at voltages of 80 to 150 volts. The higher the voltage the faster the gel runs, however the bands will be less crisp and DNA fragment sizes may be more difficult to determine. Lower voltages with longer run times tend to produce better results. A typical run time is 1–2 hours, depending on gel concentration and voltage.

7. Stop the gel when the fastest dye line (bromophenol blue) is about 80% of the way down the gel. The loading dye contains two tracking dyes, which do not stain DNA; instead they co-migrate with certain DNA fragment sizes. Bromophenol blue migrates in 1X TAE at the same rate as a 370 bp DNA fragment, and the xylene cyanol at the same rate as one 4,160 bp (4.16 kb). The gel is done when the position of these two dyes indicate good separation of the DNA fragments in the sample, but before the bromophenol blue reaches the end (to prevent small DNA fragments from migrating off the gel).

Module C—Gel Staining and Destaining (~4 hours to 16 hours [overnight])

✓ Materials

Per Gel/Student Group

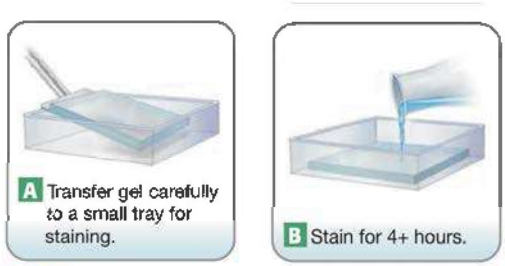
- ☐ DNA staining solution (volume to be determined by the gel and staining tray sizes.)
- ☐ Staining tray (plastic food storage containers work well)
- ☐ 0.1X TAE (volume to be determined by the gel and staining tray sizes)
- ☐ Sandwich-size zipper bag

There are many options for staining the DNA agarose gel. In research labs the most common method involves the use of ethidium bromide (EtBr), which is usually added directly to the liquid gel at a concentration of 0.2 µg/mL–0.5 µg/mL prior to casting. This allows immediate visualization of small DNA quantities under UV light without separate staining or destaining steps. EtBr stains DNA by intercalating between strands of the macromolecule and deforming its structure. Because of this effect, EtBr is suspected to be highly mutagenic and therefore is not the safest option for classroom experimentation. SYBR®-based dyes, such as SYBR® Safe or SYBR® Green are reportedly safer; however, they too physically interact with DNA structure and thus have toxic or mutagenic potential. In this protocol, we will use methylene blue, which has a long history of use in a variety of applications, including chemotherapy for various diseases.

Like other basic stains, methylene blue is just a colored molecule with a cationic chromophore that will be attracted to any negatively charged surface (Exercise 3-5). It will stain both agarose and DNA, but larger quantities of DNA will stain more intensely than the agarose, allowing visualization of the blue DNA bands on a lighter blue background. Commercially available blue staining options include the InstaStain® Blue card and Flash Blue™ DNA stain. While EtBr can detect DNA bands of 1 ng, some SYBR®–based dyes report detection levels of 60 pg. Methylene blue requires 5X the amount of DNA that would produce moderate bands with EtBr. Typically 0.5 µg to 2.5 µg of a 1 kb fragment is necessary for acceptable visibility.

Follow the procedural diagram in Figure G.4.

1. Carefully transfer the gel from the electrophoresis chamber to an appropriately sized staining tray (Fig. G.4A). Resistance to the electrical current that had been running through the gel will have generated heat, making the gel warm, a bit soft, and very slippery. Keep both hands on the ends of the casting tray to prevent the gel falling out. Gently slide the gel off the casting tray into the staining tray.
2. Add enough DNA staining solution to completely submerge the gel. Stain until DNA bands are visible in the gel. Depending on the µg quantity of DNA in your samples this will typically take 4 to 8 hours (Fig. G.4B). The gel can be left to stain overnight, if necessary.
3. Destain the gel as necessary by submerging it in 0.1X TAE, 30 minutes to 8 hours or more. If all the DNA fragments you expect to see are easily visible upon staining, destaining may not be required. (Sometimes visibility is enhanced by observing the gel on top of a white light box.) Destaining serves to remove (by diffusion) methylene blue from the agarose faster than from the DNA, enhancing contrast between the DNA and the background agarose.



G.4 Procedural Diagram for Gel Staining ♦

Destaining efficiency can be enhanced by using warm 0.1X TAE (37°C) and changing the solution when it becomes visibly blue. Keep in mind that DNA molecules will slowly diffuse in a radiating pattern from their position in the gel any time the gel is sitting in a solution. Although they do not migrate rapidly, two rounds of overnight soaking, particularly in lower percentage agarose gels, can result in the DNA bands appearing fuzzy and diffuse. Completed gels can be stored without buffer in plastic zipper bags at 4°C for many months if repeat observation is desired.

G

APPENDIX H

Medium, Reagent, and Stain Recipes

Media

General Preparation Methods

Unless otherwise specified, prepare all media as follows.

Broths

1. Suspend the dry ingredients in water. Agitate and heat slightly (if necessary) to dissolve completely.
2. Dispense 7.0 mL portions into 16 × 150 mm test tubes and cap loosely.
3. Autoclave for 15 minutes at 121°C to sterilize the medium.

Slants and Stabs

1. Suspend the dry ingredients in the water, mix well, and boil until completely dissolved.
2. Dispense 7.0 mL (or 10.0 mL for deep stabs) portions into 16 × 150 mm test tubes, and cap loosely.
3. Autoclave for 15 minutes at 121°C to sterilize the medium.
4. Slant the tubes or leave upright as needed for stabs and cool to room temperature.

Plates

1. Suspend the dry ingredients in the water, mix well, and boil until completely dissolved.
2. Cover loosely and sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
3. Remove from the autoclave, allow the medium to cool to approximately 50°C, and aseptically pour into sterile 100 mm Petri dishes (20.0 mL/plate).
4. Allow the medium to cool to room temperature. Store upside down.

Specific Recipes

Ames Test: Complete Medium

- Beef extract . . . . . 3.0 g
- Peptone . . . . . 5.0 g
- Sodium chloride . . . . . 5.0 g
- Agar . . . . . 20.0 g
- Distilled or deionized water . . . . . 1.0 L

Ames Test: Minimal Medium

- Dextrose (glucose) . . . . . 20.0 g
- 50× Vogel-Bonner salts . . . . . 20.0 mL
- Histidine . . . . . 0.00016 g
- Biotin . . . . . 0.00025 g
- Agar . . . . . 20.0 g
- Distilled or deionized water . . . . . 1.0 L

1. Prior to preparation of the medium:
   a. Add 1.6 mg histidine to 10.0 mL distilled or deionized water and filter sterilize.
   b. Add 2.5 mg biotin to 10.0 mL distilled or deionized water and filter sterilize.
2. Prepare the 50× Vogel-Bonner salts solution by adding the ingredients to just enough water to dissolve them while heating and stirring. After the ingredients are dissolved, add enough water to bring the total volume up to exactly 1 liter.
3. Suspend, mix, and boil the agar in 500.0 mL of water until completely dissolved.
4. Suspend and mix the dextrose in the remaining 500.0 mL of water until completely dissolved.
5. Cover the agar and dextrose containers loosely and sterilize in the autoclave at 121°C for 15 minutes.
6. Remove from the autoclave and cool to 80°C.
7. Aseptically add 1.0 mL histidine solution, 1.0 mL biotin solution, and 20 mL 50× Vogel-Bonner salts to the glucose solution. Mix well.
8. Add the glucose solution to the agar solution, mix well and aseptically pour into sterile Petri dishes (20 mL/plate). Allow the medium to cool to room temperature.

Ammonium Broth

- Ammonium sulfate . . . . . 1.0 g
- $MgSO_4$ . . . . . 0.5 g
- NaCl . . . . . 2.0 g
- K_2HPO_4 . . . . . 1.0 g
- $CaCO_3$ . . . . . 10.0 g
- $FeCl_3$ . . . . . trace
- Distilled or deionized water . . . . . 1.0 L

pH 7.3 at 25°C

Bile Esculin Agar (Plate or Slant)

- Beef extract . . . . . 3.0 g
- Peptone . . . . . 5.0 g
- Oxgall . . . . . 40.0 g
- Esculin . . . . . 1.0 g
- Ferric citrate . . . . . 0.5 g
- Agar . . . . . 15.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.6–6.8 at 25°C

Blood Agar

- Infusion from beef heart (solids) . . . . 2.0 g
- Pancreatic digest of casein . . . . . 13.0 g
- Sodium chloride . . . . . 5.0 g
- Yeast extract . . . . . 5.0 g
- Agar . . . . . 15.0 g
- Defibrinated sheep blood . . . . . 50.0 mL
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved. This is blood agar base.
2. Cover loosely and sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
3. Remove from the autoclave and cool to 45°C.
4. Aseptically add the sterile, room-temperature sheep blood to the blood agar base and mix well.
5. Pour into sterile Petri dishes and allow the medium to cool to room temperature.

Brain-Heart Infusion Agar (Plate or Slant)

- Calf brains, infusion from 200 g . . . . 7.7 g
- Beef heart, infusion from 250 g . . . . . 9.8 g
- Proteose peptone . . . . . 10.0 g
- Dextrose . . . . . 2.0 g
- Sodium chloride . . . . . 5.0 g
- Disodium phosphate . . . . . 2.5 g
- Agar . . . . . 15.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.2–7.6 at 25°C

Brain-Heart Infusion Broth

- Calf brains, infusion from 200 g . . . . 7.7 g
- Beef heart, infusion from 250 g . . . . . 9.8 g
- Proteose peptone . . . . . 10.0 g
- Dextrose . . . . . 2.0 g
- Sodium chloride . . . . . 5.0 g
- Disodium phosphate . . . . . 2.5 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.2–7.6 at 25°C

Brilliant Green Lactose Bile Broth

- Peptone . . . . . 10.0 g
- Lactose . . . . . 10.0 g
- Oxgall . . . . . 20.0 g
- Brilliant green dye . . . . . 0.0133 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.0–7.4 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Dispense 10.0 mL portions into test tubes.
3. Place an inverted Durham tube in each broth and cap loosely.
4. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
5. Remove the medium from the autoclave and allow it to cool before inoculating.

Citrate Agar (Simmons)

- Ammonium dihydrogen phosphate . . 1.0 g
- Dipotassium phosphate . . . . . 1.0 g
- Sodium chloride . . . . . 5.0 g
- Sodium citrate . . . . . 2.0 g
- Magnesium sulfate . . . . . 0.2 g
- Agar . . . . . 15.0 g
- Bromothymol blue . . . . . 0.08 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.7–7.1 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Dispense 7.0 mL portions into test tubes and cap loosely.
3. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
4. Remove from the autoclave and position tubes in such a way as to produce a slant nearly to the tube's base.
5. Cool to room temperature.

Columbia CNA with 5% Sheep Blood Agar

- Pancreatic digest of casein . . . . . 12.0 g
- Peptic digest of animal tissue . . . . . 5.0 g
- Yeast extract . . . . . 3.0 g
- Beef extract . . . . . 3.0 g
- Corn starch . . . . . 1.0 g
- Sodium chloride . . . . . 5.0 g
- Colistin . . . . . 10.0 mg
- Nalidixic acid . . . . . 10.0 mg
- Agar . . . . . 13.5 g
- Sheep blood (defibrinated, sterile) . . . 50.0 mL
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Cover loosely and sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
3. Remove from the autoclave and cool to 45 to 50°C.
4. Add the sheep blood to 950 mL of the mixture and aseptically pour into sterile Petri dishes (20 mL/plate).
5. Allow the medium to cool to room temperature. Store upside down.

Complete Medium (See Ames Test)

Decarboxylase Medium (Møller)

- Peptone . . . . . 5.0 g
- Beef extract . . . . . 5.0 g
- Glucose (dextrose) . . . . . 0.5 g
- Bromcresol purple . . . . . 0.01 g
- Cresol red . . . . . 0.005 g
- Pyridoxal . . . . . 0.005 g
- L-lysine, L-ornithine, or L-arginine . . 10.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 5.8–6.2 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved. (Use only one of the listed L-amino acids.)
2. Adjust pH by adding NaOH if necessary.
3. Dispense 7.0 mL volumes into test tubes and cap.
4. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 10 minutes.
5. Remove from the autoclave and cool to room temperature.

Desoxycholate Agar (Modified Leifson)

- Peptone . . . . . 10.0 g
- Lactose . . . . . 10.0 g
- Sodium desoxycholate . . . . . 1.0 g
- Sodium chloride . . . . . 5.0 g
- Dipotassium phosphate . . . . . 2.0 g
- Ferric citrate . . . . . 1.0 g
- Sodium citrate . . . . . 1.0 g
- Agar . . . . . 16.0 g
- Neutral red . . . . . 0.033 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

1. Suspend the dry ingredients in the water, mix well, and boil until completely dissolved.
2. Do not autoclave.
3. When cooled to 50°C, pour into sterile plates.
4. Allow the medium to cool to room temperature.

(Modified) *Desulfovibrio* Medium

Base Medium

- Yeast extract . . . . . 5.0 g
- Wolfe's mineral solution . . . . . 10.0 mL (See **Modified Thiobacillus Medium**)
- Ammonium phosphate, dibasic . . . 0.3 g
- Sodium lactate . . . . . 3.0 g
- Sodium sulfite . . . . . 2.0 g
- Dipotassium phosphate . . . . . 1.7 g
- L-Cysteine . . . . . 0.5 g
- Ferrous ammonium sulfate solution . . . . . 1.0 mL (0.005 g)
- Agar . . . . . 3.0 g
- DI H_2O . . . . . 1.0 L

pH 7.5 5 0.2 at 25°C

1. Mix all ingredients and boil to dissolve the agar.
2. Transfer 8 mL volumes to screw-cap test tubes and cap loosely.
3. Autoclave at 121°C for 15 minutes. (***Note:*** To ensure anaerobic conditions in the medium, it must be autoclaved immediately before class, and placed in a 50°C water bath.)

Ferrous Ammonium Sulfate Solution for Sulfur Reducing Bacteria

- Ferrous ammonium sulfate . . . . . 0.5 g
- dH_2O . . . . . 100.0 mL

1. Add the ferrous ammonium sulfate to the water and mix.
2. Filter sterilize when needed using a syringe and sterile 0.22 or 0.44 µm filter.

DNase Test Agar with Methyl Green

- Tryptose . . . . . 20.0 g
- Deoxyribonucleic acid . . . . . 2.0 g
- Sodium chloride . . . . . 5.0 g
- Agar . . . . . 15.0 g
- Methyl green . . . . . 0.05 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

EC Broth

- Tryptose . . . . . 20.0 g
- Lactose . . . . . 5.0 g
- Dipotassium phosphate . . . . . 4.0 g
- Monopotassium phosphate . . . . . 1.5 g
- Sodium chloride . . . . . 5.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.7–7.1 at 25°C

1. Suspend the dry ingredients in water, and mix well until completely dissolved.
2. Dispense 10.0 mL portions into test tubes.
3. Place an inverted Durham tube in each broth and cap loosely.
4. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
5. Remove the media from the autoclave and allow it to cool before inoculating.

Endo Agar LES

- Yeast extract . . . . . 1.2 g
- Casitone . . . . . 3.7 g
- Thiopeptone . . . . . 3.7 g
- Tryptonse . . . . . 7.5 g
- Lactose . . . . . 9.4 g
- Dipotassium phosphate . . . . . 3.3 g
- Monopotassium phosphate . . . . . 1.0 g
- Sodium chloride . . . . . 3.7 g
- Sodium desoxycholate . . . . . 0.1 g
- Sodium lauryl sulfate . . . . . 0.05 g
- Sodium sulfite . . . . . 1.6 g
- Basic fuchsin . . . . . 0.8 g
- Agar . . . . . 15.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.3–7.7 at 25°C

Enriched TSA (See Tryptic Soy Agar)

Eosin Methylene Blue Agar (Levine)

- Peptone . . . . . 10.0 g
- Lactose . . . . . 10.0 g[1]
- Dipotassium phosphate . . . . . 2.0 g
- Agar . . . . . 15.0 g
- Eosin Y . . . . . 0.4 g
- Methylene blue . . . . . 0.065 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.9–7.3 at 25°C

Glucose Broth

- Peptone . . . . . 10.0 g
- Glucose . . . . . 5.0 g
- NaCl . . . . . 5.0 g
- Distilled or deionized water . . . . . 1.0 L

Glucose Salts Medium

- Glucose . . . . . 5.0 g
- NaCl . . . . . 5.0 g
- $MgSO_4$ . . . . . 0.2 g
- $(NH_4)H_2PO_4$ . . . . . 1.0 g
- K_2HPO_4 . . . . . 1.0 g
- Distilled or deionized water . . . . . 1.0 L

Glycerol Yeast Extract Agar

- Glycerol . . . . . 5.0 mL
- Yeast extract . . . . . 2.0 g
- Dipotassium phosphate . . . . . 1.0 g
- Agar . . . . . 15.0 g
- Distilled or deionized water . . . . . 1.0 L

Hektoen Enteric Agar

- Yeast extract . . . . . 3.0 g
- Peptic digest of animal tissue . . . . . 12.0 g
- Lactose . . . . . 12.0 g
- Sucrose . . . . . 12.0 g
- Salicin . . . . . 2.0 g
- Bile salts . . . . . 9.0 g
- Sodium chloride . . . . . 5.0 g
- Sodium thiosulfate . . . . . 5.0 g
- Ferric ammonium citrate . . . . . 1.5 g
- Bromothymol blue . . . . . 0.064 g
- Acid fuchsin . . . . . 0.1 g
- Agar . . . . . 13.5 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.4–7.8 at 25°C

[1] An alternative recipe replaces the 10.0 g of lactose with 5.0 g of lactose and 5.0 g of sucrose.

1. Suspend the dry ingredients in the water, mix well, and boil until completely dissolved.
2. Do not autoclave.
3. When cooled to 50°C, pour into sterile plates.
4. Cool to room temperature with lids slightly open.

Kligler Iron Agar

- Pancreatic digest of casein . . . . . 10.0 g
- Peptic digest of animal tissue . . . . . 10.0 g
- Lactose . . . . . 10.0 g
- Dextrose (glucose) . . . . . 1.0 g
- Ferric ammonium citrate . . . . . 0.5 g
- Sodium chloride . . . . . 5.0 g
- Sodium thiosulfate . . . . . 0.5 g
- Agar . . . . . 15.0 g
- Phenol red . . . . . 0.025 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.2–7.6 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Transfer 7.0 mL portions to test tubes and cap loosely.
3. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
4. Remove from the autoclave and slant in such a way as to form a deep butt.
5. Allow the medium to cool to room temperature.

Lactobacilli MRS Agar

- Proteose peptone no. 3 . . . . . 10.0 g
- Beef extract . . . . . 10.0 g
- Yeast extract . . . . . 5.0 g
- Dextrose . . . . . 20.0 g
- Polysorbate 80 . . . . . 1.0 g
- Ammonium citrate . . . . . 2.0 g
- Sodium acetate . . . . . 5.0 g
- Magnesium sulfate . . . . . 0.1 g
- Manganese sulfate . . . . . 0.05 g
- Dipotassium phosphate . . . . . 2.0 g
- Distilled or deionized water . . . . . 1.0 L
- Agar . . . . . 15.0 g

pH 6.3–6.7 at 25°C

Lactobacilli MRS Broth

- Proteose peptone no. 3 . . . . . 10.0 g
- Beef extract . . . . . 10.0 g
- Yeast extract . . . . . 5.0 g
- Dextrose . . . . . 20.0 g
- Polysorbate 80 . . . . . 1.0 g
- Ammonium citrate . . . . . 2.0 g
- Sodium acetate . . . . . 5.0 g
- Magnesium sulfate . . . . . 0.1 g
- Manganese sulfate . . . . . 0.05 g
- Dipotassium phosphate . . . . . 2.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.3–6.7 at 25°C

Lauryl Tryptose Broth

- Tryptose . . . . . 20.0 g
- Lactose . . . . . 5.0 g
- Dipotassium phosphate . . . . . 2.75 g
- Monopotassium phosphate . . . . . 2.75 g
- Sodium chloride . . . . . 5.0 g
- Sodium lauryl sulfate . . . . . 0.1 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.6–7.0 at 25°C

1. Suspend the dry ingredients in water until completely dissolved. Heat slightly if necessary.
2. Dispense 10.0 mL portions into test tubes.
3. Place an inverted Durham tube in each broth and cap loosely.
4. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
5. Remove the medium from the autoclave and allow it to cool before inoculating.

Litmus Milk Medium

- Skim milk . . . . . 100.0 g
- Azolitmin . . . . . 0.5 g
- Sodium sulfite . . . . . 0.5 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.3–6.7 at 25°C

1. Suspend and mix the ingredients in water and heat to approximately 50°C to dissolve completely.
2. Transfer 7.0 mL portions to test tubes and cap loosely.
3. Sterilize in the autoclave at 113°C–115°C for 20 minutes.
4. Remove from the autoclave and allow the medium to cool to room temperature.

Luria-Bertani Agar

- Tryptone . . . . . 10.0 g
- Yeast extract . . . . . 5.0 g
- NaCl . . . . . 10.0 g
- Agar . . . . . 15.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.4 at 25°C

Luria-Bertani Broth

- Tryptone . . . . . 10.0 g
- Yeast extract . . . . . 5.0 g
- NaCl . . . . . 10.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.4 at 25°C

Lysine Iron Agar

- Peptone . . . . . 5.0 g
- Yeast extract . . . . . 3.0 g
- Dextrose . . . . . 1.0 g
- L-lysine hydrochloride . . . . . 10.0 g
- Ferric ammonium citrate . . . . . 0.5 g
- Sodium thiosulfate . . . . . 0.04 g
- Bromcresol purple . . . . . 0.02 g
- Agar . . . . . 15.0 g

pH 6.5–6.9 at 25°C

1. Suspend the dry ingredients in the water, mix well, and boil until completely dissolved.
2. Transfer 8.0 mL portions to test tubes and cap loosely.
3. Sterilize in the autoclave at 121°C for 15 minutes.
4. Remove from the autoclave and slant in such a way as to produce a deep butt.
5. Allow the medium to cool to room temperature.

H

MacConkey Agar

- Pancreatic digest of gelatin . . . . . 17.0 g
- Pancreatic digest of casein . . . . . 1.5 g
- Peptic digest of animal tissue . . . . . 1.5 g
- Lactose . . . . . 10.0 g
- Bile salts . . . . . 1.5 g
- Sodium chloride . . . . . 5.0 g
- Neutral red . . . . . 0.03 g
- Crystal violet . . . . . 0.001 g
- Agar . . . . . 13.5 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.9–7.3 at 25°C

Malonate Broth

- Yeast extract . . . . . 1.0 g
- Ammonium sulfate . . . . . 2.0 g
- Dipotassium phosphate . . . . . 0.6 g
- Monopotassium phosphate . . . . . 0.4 g
- Sodium chloride . . . . . 2.0 g
- Sodium malonate . . . . . 3.0 g
- Dextrose . . . . . 0.25 g
- Bromothymol blue . . . . . 0.025 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.5–6.9 at 25°C

Mannitol Salt Agar

- Beef extract . . . . . 1.0 g
- Peptone . . . . . 10.0 g
- Sodium chloride . . . . . 75.0 g
- D-Mannitol . . . . . 10.0 g
- Phenol red . . . . . 0.025 g
- Agar . . . . . 15.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.2–7.6 at 25°C

Microgradient Tubes

Top Layer (Mineral Agar)

- Basal medium . . . . . 1.0 L
- Wolfe's vitamin mix . . . . . 1.0 mL
- Agar . . . . . 2.5 g

Bottom Layer (Plug)[2]

- Basal medium . . . . . 1.0 L
- $Na_2S_2O_3$ . . . . . 15.0 g
- Agar . . . . . 15.0 g

Basal Medium

- Wolfe's mineral solution . . . . . 10.0 mL
- 1M K_2HPO_4 . . . . . 5.7 mL
- 1M KH_2PO_4 . . . . . 3.3 mL
- 1M $NaHCO_3$ . . . . . 10.0 mL
- 1M $(NH_4)_2SO_4$ . . . . . 9.0 mL
- 1M $MgCl_2$ . . . . . 1.0 mL
- 1M $CaCl_2$ . . . . . 0.5 mL
- NaCl . . . . . 1.0 g
- KCl . . . . . 0.1 g
- dIH_2O . . . . . to 1.0 L

pH adjusted to 7

1. Add 500 mL of basal medium to two flasks labeled "mineral agar" and "plug."

[2] Due to the volatility and toxicity of sulfide gas, $Na_2S_2O_3$ is substituted for Na_2S.

2 Add 1.25 g of agar to the mineral agar flask. Cover and boil to dissolve the agar.

3 To the plug flask, add 7.5 g $Na_2S_2O_3$ and 7.5 g of agar. Cover and boil to dissolve the agar.

4 Transfer ~1 mL of the plug mix to medium size test tubes and cap loosely.

5 Autoclave all tubes and the flask containing the mineral agar mixture at 121°C for 15 minutes.

6 When finished autoclaving, place the flask in a 50°C water bath and allow the temperatures to equilibrate.

7 Place the tubes in a test tube rack in an ice-water bath.

8 When the mineral agar has cooled to 50°C, add 0.5 mL of Wolfe's vitamin solution and mix.

9 When the plugs have solidified and cooled, pour (or pipette) ~ 9 mL volumes of the molten mineral agar into each tube. Keep the tubes cold so as not to loosen or melt the plugs. Try to avoid creating bubbles. The final plug to mineral agar ratio should be 1:9.

(Skim) Milk Agar

- Beef extract . . . . . 3.0 g
- Peptone . . . . . 5.0 g
- Agar . . . . . 15.0 g
- Powdered nonfat milk . . . . . 100.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.0–7.4 at 25°C

1 Suspend the powdered milk in 500.0 mL of water in a 1-liter flask, mix well, and cover loosely.

2 Suspend the remainder of the ingredients in 500.0 mL of water in a 1-liter flask, mix well, boil to dissolve completely, and cover loosely.

3 Sterilize in the autoclave at 113–115°C for 20 minutes.

4 Remove from the autoclave and allow the mixtures to cool slightly.

5 Aseptically pour the agar solution into the milk solution. Mix *gently* (to prevent foaming).

6 Aseptically pour into sterile Petri dishes (15 mL/plate).

7 Allow the medium to cool to room temperature.

Minimal Medium (See Ames Test)

Modified *Desulfovibrio* Medium
(See *Desulfovibrio* Medium)

Modified *Thiobacillus* Medium
(See *Thiobacillus* Medium)

Motility Test Medium

- Beef extract . . . . . 3.0 g
- Pancreatic digest of gelatin . . . . . 10.0 g
- Sodium chloride . . . . . 5.0 g
- Agar . . . . . 4.0 g
- Triphenyltetrazolium chloride (TTC) . . . . . 0.05 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

MR-VP Broth

- Buffered peptone . . . . . 7.0 g
- Dipotassium phosphate . . . . . 5.0 g
- Dextrose (glucose) . . . . . 5.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.7–7.1 at 25°C

Mueller-Hinton II Agar

- Beef extract . . . . . 2.0 g
- Acid hydrolysate of casein . . . . . 17.5 g
- Starch . . . . . 1.5 g
- Agar . . . . . 17.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

1 Suspend the dry ingredients in water, mix well, and boil until completely dissolved.

2 Cover loosely and sterilize in the autoclave at 121°C (15 lbs.) for 15 minutes.

3 Remove from the autoclave, cool to approximately 50°C.

4 Aseptically pour into sterile Petri dishes to a depth of 4 mm.

5 Allow the medium to cool to room temperature.

Nitrate Broth

- Beef extract . . . . . 3.0 g
- Peptone . . . . . 5.0 g
- Potassium nitrate . . . . . 1.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.6–7.0 at 25°C

1 Suspend the ingredients in water; mix well, and warm until completely dissolved.

2 Transfer 8.0 mL portions to test tubes, and cap loosely. (Add inverted Durham tubes before capping, if desired.)

3 Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.

4 Remove from the autoclave and allow the medium to cool to room temperature.

Nitrogen Free Broth

- Glucose ..... 10.0 g
- $CaCO_3$ ..... 5.0 g
- $CaCl_2$ ..... 0.1 g
- KH_2PO_4 ..... 0.1 g
- K_2HPO_4 ..... 0.9 g
- $MgSO_4$ ..... 0.1 g
- $FeSO_4$ ..... 10.0 mg
- Na_2MoO_4 ..... 5.0 mg
- Distilled or deionized water ..... 1.0 L

pH 7.3 at 25°C

Nutrient Agar (Plate or Tube)

- Beef extract ..... 3.0 g
- Peptone ..... 5.0 g
- Agar ..... 15.0 g
- Distilled or deionized water ..... 1.0 L

pH 6.6–7.0 at 25°C

NA Tubes

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Dispense 7 mL or 10 mL portions into test tubes, and cap loosely.
3. Autoclave for 15 minutes at 121°C to sterilize the medium.
4. Cool to room temperature with the tubes in an upright position (10 mL) for agar deep tubes. Cool with the tubes on an angle (7 mL) for agar slants.

Nutrient Broth

- Beef extract ..... 3.0 g
- Peptone ..... 5.0 g
- Distilled or deionized water ..... 1.0 L

pH 6.6–7.0 at 25°C

Nutrient Broth w/thiosulfate (Flasks for Reduction Potential)

- Beef extract ..... 3.0 g
- Peptone ..... 5.0 g
- Sodium thiosulfate ..... 0.3 g
- Distilled or deionized water ..... 1.0 L

1. Suspend the dry ingredients in water. Agitate and heat slightly (if necessary) to dissolve completely.
2. Dispense 15.0 mL portions into 25 mL flasks, and cap loosely.
3. Autoclave for 15 minutes at 121°C to sterilize the medium.

Nutrient Gelatin

- Beef extract ..... 3.0 g
- Peptone ..... 5.0 g
- Gelatin ..... 120.0 g
- Distilled or deionized water ..... 1.0 L

pH 6.6–7.0 at 25°C

1. *Slowly* add the dry ingredients to the water while stirring.
2. Warm to >50°C and maintain temperature until completely dissolved.
3. Dispense 7.0 mL volumes into test tubes and cap loosely.
4. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
5. Remove from the autoclave immediately and allow the medium to cool to room temperature in the upright position.

O-F Medium (Hugh and Leifson)

Basal Medium

- Pancreatic digest of casein ..... 2.0 g
- Sodium chloride ..... 5.0 g
- Dipotassium phosphate ..... 0.3 g
- Agar ..... 2.5 g
- Bromothymol blue ..... 0.03 g
- Distilled or deionized water ..... 1.0 L

pH 6.6–7.0 at 25°C

O-F Carbohydrate Solution

- Carbohydrate (glucose, lactose, sucrose) ..... 1.1 g
- Distilled or deionized water to total ..... 10.0 mL

1. Suspend the dry basal medium ingredients, *without the carbohydrate*, in water, mix well, and boil to dissolve completely. This is basal medium.
2. Divide the medium into 10 aliquots of 100.0 mL each.
3. Cover loosely and sterilize in the autoclave at 121°C for 15 minutes.
4. Prepare carbohydrate solution, cover loosely, and autoclave at 118°C for 10 minutes.
5. Allow both solutions to cool to 50°C.
6. Aseptically add 10.0 mL sterile carbohydrate solution to a basal medium aliquot, and mix well.
7. Aseptically transfer 7.0 mL volumes to sterile test tubes, and allow the medium to cool to room temperature.

Peptone (Broth) Water

- Peptone . . . . . 40.0 g
- Distilled or deionized water . . . . . 1.0 L

Phenol Red (Carbohydrate) Broth

- Pancreatic digest of casein . . . . . 10.0 g
- Sodium chloride . . . . . 5.0 g
- Carbohydrate (use any; e.g., glucose, lactose, sucrose, etc.) . . . . 5.0 g
- Phenol red . . . . . 0.018 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.157.5 at 25°C

1. Suspend the dry ingredients in water; mix well, and warm slightly to dissolve completely.
2. Dispense 7.0 mL volumes into test tubes.
3. Insert inverted Durham tubes into the test tubes and cap loosely.
4. Sterilize in the autoclave at 116–118°C for 15 minutes.
5. Remove from the autoclave and allow the medium to cool to room temperature.

Phenylalanine Deaminase Agar

- DL-Phenylalanine . . . . . 2.0 g
- Yeast extract . . . . . 3.0 g
- Sodium chloride . . . . . 5.0 g
- Sodium phosphate . . . . . 1.0 g
- Agar . . . . . 12.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Dispense 7.0 mL volumes into test tubes and cap loosely.
3. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 10 minutes.
4. Remove from the autoclave, slant, and allow the medium to cool to room temperature.

Phenylethyl Alcohol Agar

- Pancreatic digest of casein . . . . . 15.0 g
- Papaic digest of soybean meal . . . . . 5.0 g
- Sodium chloride . . . . . 5.0 g
- β-Phenylethyl alcohol . . . . . 2.5 g
- Agar . . . . . 15.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

Photobacterium Broth

- Tryptone . . . . . 5.0 g
- Yeast extract . . . . . 2.5 g
- Ammonium chloride . . . . . 0.3 g
- Magnesium sulfate . . . . . 0.3 g
- Ferric chloride . . . . . 0.01 g
- Calcium carbonate . . . . . 1.0 g
- Monopotassium phosphate . . . . . 3.0 g
- Sodium glycerol phosphate . . . . . 23.5 g
- Sodium chloride . . . . . 30.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.8–7.2 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Dispense into flasks to form a shallow layer.
3. Autoclave for 15 minutes at 15 lbs. pressure (121°C).
4. Allow the medium to cool to room temperature.

Potato Dextrose Agar

- Potato flakes . . . . . 20.0 g
- Dextrose . . . . . 10.0 g
- Agar . . . . . 15.0 g
- Distilled or deionized water . . . . . 1.0 L

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Autoclave for 15 minutes at 15 lbs. pressure (121°C).
3. Remove the agar mixture from the autoclave and cool to 50°C.
4. Mix and pour into sterile Petri dishes. ***Note:*** Swirl the flask frequently to keep the flakes uniformly distributed.

Purple Broth

- Peptone . . . . . 10.0 g
- Beef extract . . . . . 1.0 g
- Sodium chloride . . . . . 5.0 g
- Bromcresol purple . . . . . 0.02 g
- Carbohydrate (use any; e.g., glucose, lactose, sucrose, etc.) . . . 10.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.6–7.0 at 25°C

1. Suspend the dry ingredients in water, mix well, and warm slightly to dissolve completely.
2. Dispense 9.0 mL volumes into test tubes.
3. Insert inverted Durham tubes into the test tubes and cap loosely.
4. Sterilize in the autoclave at 118°C for 15 minutes.
5. Remove from the autoclave and allow the medium to cool to room temperature.

Sabouraud Dextrose Agar

(with antibiotics added to inhibit bacterial growth)

- Peptone . . . . . 10.0 g
- Dextrose . . . . . 40.0 g
- Agar . . . . . 15.0 g
- Penicillin[3] . . . . . 20,000.0 units
- Streptomycin[3] . . . . . 0.00004 g
- Distilled or deionized water . . . . . 1.0 L

pH 5.2–5.6 at 25°C

1. Suspend the peptone, dextrose, and agar in water, mix well, and boil until completely dissolved.
2. Autoclave for 15 minutes at 15 lbs. pressure (121°C).
3. Remove the agar mixture from the autoclave and cool to 50°C.
4. Aseptically add antibiotics. Mix and pour into sterile Petri dishes.
5. Allow the medium to cool to room temperature.

Saline Agar: Double Gel Immunodiffusion

- Sodium chloride . . . . . 10.0 g
- Agar . . . . . 20.0 g
- Distilled or deionized water . . . . . 1.0 L

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Pour into Petri dishes to a depth of 3 mm. Do not replace the lids until the agar has solidified and cooled to room temperature.

Seawater Agar

- Peptone . . . . . 5.0 g
- Yeast extract . . . . . 5.0 g
- Beef extract . . . . . 3.0 g
- Agar . . . . . 15.0 g
- Seawater (synthetic) . . . . . 1.0 L

H

1. Suspend the ingredients in seawater, mix well, and boil to dissolve completely.
2. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
3. When cooled to 50°C, pour into sterile plates.
4. Allow the medium to cool to room temperature.

[3] To obtain the desired proportions of antibiotics in the medium, prepare as follows:

1. Dissolve 100,000 units penicillin in 10 mL sterile distilled or deionized water. Add 2 mL to 1.0 liter of agar medium.
2. Dissolve 1.0 g streptomycin in 10 mL sterile distilled or deionized water. Add 1.0 mL of this mixture to 9.0 mL sterile distilled or deionized water. Add 4 mL of this diluted mixture to 1.0 liter of agar medium.

SIM (Sulfur-Indole-Motility) Medium

- Pancreatic digest of casein . . . . . 20.0 g
- Peptic digest of animal tissue . . . . . 6.1 g
- Ferrous ammonium sulfate . . . . . 0.2 g
- Sodium thiosulfate . . . . . 0.2 g
- Agar . . . . . 3.5 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

Skim Milk Agar (See [Skim] Milk Agar)

Snyder Test Medium

- Pancreatic digest of casein . . . . . 13.5 g
- Yeast extract . . . . . 6.5 g
- Dextrose . . . . . 20.0 g
- Sodium chloride . . . . . 5.0 g
- Agar . . . . . 16.0 g
- Bromcresol green . . . . . 0.02 g
- Distilled or deionized water . . . . . 1.0 L

pH 4.6–5.0 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Transfer 7.0 mL portions to test tubes and cap loosely.
3. Sterilize in the autoclave at 118°C–121°C for 15 minutes.
4. Remove from the autoclave and place in a hot water bath set at 45°C–50°C. Allow at least 30 minutes for the agar temperature to equilibrate before beginning the exercise.

Soft Agar

- Beef extract . . . . . 3.0 g
- Peptone . . . . . 5.0 g
- Sodium chloride . . . . . 5.0 g
- Tryptone . . . . . 2.5 g
- Yeast extract . . . . . 2.5 g
- Agar . . . . . 7.0 g
- Distilled or deionized water . . . . . 1.0 L

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Transfer 2.5 mL portions to test tubes and cap loosely.
3. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
4. Remove from the autoclave and place in a hot water bath set at 45°C. Allow 30 minutes for the agar temperature to equilibrate.

Sporulating Agar

- Pancreatic digest of gelatin . . . . . 6.0 g
- Pancreatic digest of casein . . . . . 4.0 g
- Yeast extract . . . . . 3.0 g
- Beef extract . . . . . 1.5 g
- Dextrose . . . . . 1.0 g
- Agar . . . . . 15.0 g
- Manganous sulfate . . . . . 0.3 g
- Distilled or deionized water . . . . . 1.0 L

Final ph 6.6 5 0.2 at 25°C

Starch Agar

- Beef extract . . . . . 3.0 g
- Soluble starch . . . . . 10.0 g
- Agar . . . . . 12.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.3–7.7 at 25°C

(Modified) *Thiobacillus* Medium

Base Medium

- $Na_2S_2O_3$ . . . . . 10.0 g
- Na_2HPO_4 . . . . . 1.2 g
- KH_2PO_4 . . . . . 1.8 g
- $(NH_4)_2SO_4$ . . . . . 0.1 g
- $MgSO_4 \times 7H_2O$ . . . . . 0.1 g
- $CaCl_2$ . . . . . 0.03 g
- $FeCl_3$ . . . . . 0.02 g
- $MnSO_4$ . . . . . 0.02 g
- $NaHCO_3$ . . . . . 0.5 g
- KNO_3 . . . . . 5.0 g
- Wolfe's mineral solution . . . . . 10.0 mL
- Wolfe's vitamin solution (filter sterilized and added after autoclaving) . . . . 1.0 mL
- Distilled or deionized H_2O . . . . . to 1.0 L

pH 7.5 adjusted with KOH

1. Add 990 mL dH_20 to a 2 L flask.
2. Add all compounds except the vitamin solution. Mix well.
3. Adjust the pH to 7.5.
4. Autoclave at 121°C for 20 minutes.
5. Cool to approximately 50°C and add filter-sterilized vitamin solution.

Wolfe's Mineral Solution

- Nitrilotriacetic acid . . . . . 1.5 g
- $MgSO_4 \times 7H_2O$ . . . . . 3.0 g
- $MnSO_4 \times H_2O$ . . . . . 0.5 g
- NaCl . . . . . 1.0 g
- $FeSO_4 \times 7H_2O$ . . . . . 0.1 g
- $CoCl_2 \times 6H_2O$ . . . . . 0.1 g
- $CaCl_2$ . . . . . 0.1 g
- $ZnSO_4 \times 7H_2O$ . . . . . 0.1 g
- $CuSO_4 \times 5H_2O$ . . . . . 0.01 g
- $AlK(SO_4)2 \times 12H_2O$ . . . . . 0.01 g
- H_3BO_3 . . . . . 0.01 g
- $Na_2MoO_4 \times 2H_2O$ . . . . . 0.01 g
- dH_2O . . . . . 1.0 L

1. Add nitrilotriacetic acid to ~500 mL of dH_2O and adjust to pH 6.5 with KOH.
2. Bring volume to 1.0 L.
3. Add the remaining ingredients one at a time and mix well.
4. Autoclave at 121°C for 20 minutes.
5. Cool and store refrigerated in a glass or nalgene reagent bottle.

Wolfe's Vitamin Solution

(This solution must be filter sterilized, therefore reduce proportions as necessary for easier handling.)

- Biotin . . . . . 2.0 mg
- Folic acid . . . . . 2.0 mg
- Pyridoxine hydrochloride . . . . . 10.0 mg
- Thiamine–HCl . . . . . 5.0 mg
- Riboflavin . . . . . 5.0 mg
- Nicotinic acid . . . . . 5.0 mg
- Calcium D– (+) –pantothenate . . . . . 5.0 mg
- Vitamin B_{12} . . . . . 0.1 mg
- p-Aminobenzoic acid . . . . . 5.0 mg
- Thioctic acid . . . . . 5.0 mg
- dH_2O . . . . . 1.0 L

1. Add ingredients one at a time to 1.0 L distilled or deionized water and mix well.
2. Filter sterilize and store in the refrigerator.

Thioglycollate Medium (Fluid)

- Yeast extract . . . . . 5.0 g
- Casitone . . . . . 15.0 g
- Dextrose (glucose) . . . . . 5.5 g
- Sodium chloride . . . . . 2.5 g
- Sodium thioglycolate . . . . . 0.5 g
- L-Cystine . . . . . 0.5 g
- Agar . . . . . 0.75 g
- Resazurin . . . . . 0.001 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Dispense 10.0 mL into sterile screw-cap test tubes.
3. Autoclave for 15 minutes at 15 lbs. pressure (121°C) to sterilize. Allow the medium to cool to room temperature before inoculating.

Tributyrin Agar

- Beef extract . . . . . 3.0 g
- Peptone . . . . . 5.0 g
- Agar . . . . . 15.0 g
- Tributyrin oil . . . . . 10.0 mL
- Distilled or deionized water . . . . . 1.0 L

pH 5.8–6.2 at 25°C

1. Suspend the dry ingredients in water, mix well, and boil until completely dissolved.
2. Cover loosely, and sterilize together with the tube of tributyrin oil in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
3. Remove from the autoclave, and aseptically pour agar mixture into a sterile glass blender.
4. Aseptically add the tributyrin oil to the agar mixture and blend on "High" for 1 minute.
5. Aseptically pour into sterile Petri dishes (20 mL/plate). Allow the medium to cool to room temperature.

Triple Sugar Iron Agar (TSIA)

- Pancreatic digest of casein . . . . . 10.0 g
- Peptic digest of animal tissue . . . . . 10.0 g
- Dextrose (glucose) . . . . . 1.0 g
- Lactose . . . . . 10.0 g
- Sucrose . . . . . 10.0 g
- Ferrous ammonium sulfate . . . . . 0.2 g
- Sodium chloride . . . . . 5.0 g
- Sodium thiosulfate . . . . . 0.2 g
- Agar . . . . . 13.0 g
- Phenol red . . . . . 0.025 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

1. Suspend the dry ingredients in the water, mix well, and boil until completely dissolved.
2. Transfer 7.0 mL portions to test tubes and cap loosely.
3. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
4. Remove from the autoclave and slant in such a way as to form a deep butt.
5. Allow the medium to cool to room temperature.

Tryptic Nitrate Medium

- Tryptose . . . . . 20.0 g
- Dextrose . . . . . 1.0 g
- Disodium phosphate . . . . . 2.0 g
- Potassium nitrate . . . . . 1.0 g
- Agar . . . . . 1.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.0–7.4 at 25°C

1. Suspend the dry ingredients in the water, mix well, and boil until completely dissolved.
2. Transfer 10.0 mL portions to test tubes, and cap loosely.
3. Sterilize in the autoclave at 15 lbs. pressure (121°C) for 15 minutes.
4. Remove from the autoclave and allow the medium to cool to room temperature.

Tryptic Soy Agar (Plate or Tube)

- Tryptone . . . . . 15.0 g
- Soytone . . . . . 5.0 g
- Sodium chloride . . . . . 5.0 g
- Agar . . . . . 15.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

Tryptic Soy Agar (Enriched with Yeast Extract)

- Tryptone . . . . . 15.0 g
- Soytone . . . . . 5.0 g
- Sodium chloride . . . . . 5.0 g
- Yeast extract . . . . . 5.0 g
- Agar . . . . . 15.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

1. Suspend the dry ingredients in the water, mix well, and boil until completely dissolved.
2. Transfer 10.0 mL portions to test tubes and cap loosely.
3. Sterilize in the autoclave at 121°C for 15 minutes.
4. Allow the medium to cool to room temperature with the tubes in an upright position (to be used for agar stabs).

Tryptic Soy Broth

- Tryptone . . . . . 17.0 g
- Soytone . . . . . 3.0 g
- Sodium chloride . . . . . 5.0 g
- Dipotassium phosphate . . . . . 2.5 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.1–7.5 at 25°C

Tryptic Soy Broth Plus 1% Glucose

Tryptic Soy Broth (see Tryptic Soy Broth recipe)

- Glucose . . . . . 10 g

Urea Agar

- Peptone . . . . . 1.0 g
- Dextrose (glucose) . . . . . 1.0 g
- Sodium chloride . . . . . 5.0 g
- Potassium phosphate, monobasic . . . 2.0 g
- Agar . . . . . 15.0 g
- Phenol red . . . . . 0.012 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.6–7.0 at 25°C

1. Suspend the agar in 900 mL of the water, mix well, and boil to dissolve completely.
2. Cover loosely and sterilize by autoclaving at 15 lbs. pressure (121°C) for 15 minutes.
3. Remove from the autoclave and cool to 55°C.
4. Suspend the remaining ingredients in 100 mL of the water, mix well, and filter sterilize. *Do not autoclave.* This is urea agar base.
5. Aseptically add the urea agar base to the agar solution and mix well.
6. Aseptically transfer 7.0 mL portions to sterile test tubes, and cap loosely.
7. Slant in such a way that the agar butt is approximately twice as long as the slant.
8. Allow to cool to room temperature.

Urea Broth (Rustigian and Stuart)

- Yeast extract . . . . . 0.1 g
- Potassium phosphate, monobasic . . . 9.1 g
- Potassium phosphate, dibasic . . . . . 9.5 g
- Urea . . . . . 20.0 g
- Phenol red . . . . . 0.01 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.6–7.0 at 25°C

1. Suspend the dry ingredients in the water and mix well.
2. Filter sterilize the solution. *Do not autoclave.*
3. Aseptically transfer 1.0 mL volumes to small sterile test tubes and cap loosely.

Xylose Lysine Desoxycholate Agar

- Xylose . . . . . 3.5 g
- L-Lysine . . . . . 5.0 g
- Lactose . . . . . 7.5 g
- Sucrose . . . . . 7.5 g
- Sodium chloride . . . . . 5.0 g
- Yeast extract . . . . . 3.0 g
- Phenol red . . . . . 0.08 g
- Sodium desoxycholate . . . . . 2.5 g
- Sodium thiosulfate . . . . . 6.8 g
- Ferric ammonium citrate . . . . . 0.8 g
- Agar . . . . . 13.5 g
- Distilled or deionized water . . . . . 1.0 L

pH 7.3–7.7 at 25°C

1. Suspend the dry ingredients in water and mix. Heat only until the medium boils.
2. Cool in a water bath at 50°C.
3. When cooled, pour into sterile plates.
4. Allow the medium to cool to room temperature.

Yeast Extract Broth

- Beef extract . . . . . 3.0 g
- Peptone . . . . . 5.0 g
- NaCl . . . . . 5.0 g
- Yeast extract . . . . . 5.0 g
- Distilled or deionized water . . . . . 1.0 L

pH 6.6–7.0 at 25°C

Reagents

Direct Count—Staining/Diluting Agents
(See Stains)

Lysozyme Buffer

- NaCl ..... 22.67 g
- Na_2HPO_4 ..... 3.56 g
- KH_2PO_4 ..... 6.65 g
- Distilled or deionized water ..... 1.0 L

1 Dissolve all ingredients in approximately 900 mL water.

2 Add water to bring the total volume up to 1,000 mL.

Lysozyme Substrate

- Lysozyme Buffer ..... 200.0 mL
- *Micrococcus lysodeikticus* (freeze-dried) ..... 0.1 g

1 Add the freeze-dried *Micrococcus lysodeikticus* to the lysozyme buffer and mix well.

2 Using a spectrophotometer with the wavelength set at 540 nm, adjust the solution's light transmittance to 10% by adding water or *M. lysodeikticus* as needed.

MR-VP (Methyl Red–Voges-Proskauer) Test Reagents

Methyl Red

- Methyl red dye ..... 0.1 g
- Ethanol ..... 300.0 mL
- Distilled water to bring volume to ..... 500.0 mL

1 Dissolve the dye in the ethanol.

2 Add water to bring the total volume up to 500 mL.

VP Reagent A (Barritt's)

- β-naphthol ..... 5.0 g
- Absolute ethanol to bring volume to ..... 100.0 mL

1 Dissolve the β-naphthol in approximately 95 mL of ethanol.

2 Add ethanol to bring the total volume up to 100 mL.

VP Reagent B (Barritt's)

- Potassium hydroxide ..... 40.0 g
- Distilled water to bring volume to ..... 100.0 mL

1 Dissolve the potassium hydroxide in approximately 60 mL of water. (*Caution:* This solution is highly concentrated and will become hot as the KOH dissolves. It should be prepared in appropriate glassware on a stirring hot plate.) Allow it to cool to room temperature.

2 Add water to bring the volume up to 100 mL.

Methyl Red (See MR-VP)

Methylene Blue Reductase Reagent

- Methylene blue dye ..... 8.8 mg
- Distilled or deionized water ..... 200.0 mL

Nitrate Test Reagents

Reagent A

- Sulfanilic acid ..... 1.0 g
- 5N Acetic acid ..... 125.0 mL

Reagent B

- Dimethyl-β-naphthylamine ..... 1.0 g
- 5N Acetic acid ..... 200.0 mL

Oxidase Test Reagent (Kovac's)

- Tetramethyl-*p*-phenylenediamine dihydrochloride ..... 1.0 g
- Distilled or deionized water ..... 100.0 mL

Phenylalanine Deaminase Test Reagent

- Ferric chloride ..... 10.0 g
- Distilled or deionized water ..... ≈90.0 mL

1 Dissolve the ferric chloride in approximately 90 mL of distilled or deionized water.

2 Add water to bring the total volume up to 100 mL.

Voges-Proskauer Reagents A and B (See MR-VP)

Solutions

6× DNA Loading Dye (Electrophoresis)

- Glycerol ..... 30 mL (30% v/v)
- Bromophenol blue ..... 25 mg (0.25% w/v)
- Xylene cyanol FF ..... 25 mg (0.25% w/v)
- ddH_2O ..... 70 mL

DNA Staining Solution (Electrophoresis)

- Methylene blue ..... 2 mg
- 0.1× TAE buffer ..... 100 mL

McFarland Turbidity Standard (0.5)

- Barium chloride ($BaCl_2 \cdot 2H_2O$) ..... 1.175 g
- Sulfuric acid, concentrated (H_2SO_4) ..... 1.0 mL
- Distilled or deionized water ..... ≈200.0 mL

1 Pour approximately 90 mL of water into a small Erlenmeyer flask.

2 Add the $BaCl_2$ and mix well.

3 Remeasure and add water to bring the total volume up to 100 mL.

4 Add the H_2SO_4 to approximately 90 mL of water.

5 Remeasure and add water to bring the total volume up to 100 mL.

6 Add 0.5 mL of the $BaCl_2$ solution to 99.5 mL of H_2SO_4 and mix well.

7 While keeping the solution well mixed (the barium sulfate will precipitate and settle out) distribute 7 mL to 10 mL volumes into very clean screw-cap test tubes.

10× Phosphate Buffered Saline (PBS) Stock Solution

- Na_2HPO_4, anhydrous, reagent grade . . . . . 12.36 g
- $NaH_2PO_4 \times H_2O$, reagent grade . . . 1.80 g
- NaCl, reagent grade . . . . . 85.00 g

Dissolve ingredients in distilled water to a final volume of 1.0 L.

1× PBS Working solution (0.01 M phosphate, pH 7.6)

- 10× PBS Stock solution . . . . . 100 mL
- dH_2O . . . . . 900 mL

10× Tris-Acetate-EDTA (TAE) buffer

- Trisma base . . . . . 48.4 g
- Glacial acetic acid . . . . . 11.42 mL
- 0.5 M EDTA, pH 8.0 . . . . . 20.0 mL
- Deionized water . . . . . 1.0 L

1× Tris-Acetate-EDTA (TAE) buffer

- 10× TAE buffer . . . . . 100 mL
- Deionized water . . . . . 900 mL

0.1× Tris-Acetate-EDTA (TAE) buffer

- 1× TAE buffer . . . . . 100 mL
- Deionized water . . . . . 900 mL

Vogel-Bonner Salts (50×)

- Magnesium sulfate . . . . . 10.0 g
- Citric acid . . . . . 100.0 g
- Dipotassium phosphate . . . . . 500.0 g
- Monosodium ammonium phosphate . . . . . 175.0 g
- Distilled or deionized water to bring volume to 1.0 liter[4]

[4] The solid ingredients raise the solvent level significantly in this preparation. Use only enough water initially to dissolve the ingredients, and then add water to bring the volume to 1 liter.

Stains

Acid-Fast Stain, Modified Kinyoun Procedure

Carbolfuchsin (Primary Stain)

- Basic fuchsin 1.5g
- Phenol . . . . . 4.5 g
- Ethanol (95%) . . . . . 5.0 mL
- Isopropanol . . . . . 20.0 mL
- Distilled or deionized water . . . . . 75.0 mL

1 Dissolve the basic fuchsin in the ethanol and add the isopropanol.

2 Mix the phenol in the water.

3 Mix the solutions together and let stand for several days.

4 Filter before use.

Decolorizer

- H_2SO_4 . . . . . 1.0 mL
- Ethanol (95%) . . . . . 70.0 mL
- Distilled or deionized water . . . . . 29.0 mL

Brilliant Green (Counterstain)

- Brilliant green dye . . . . . 1.0 g
- Sodium azide 0.01g
- Distilled or deionized water . . . . . 100.0 mL

Acid-Fast Stain, Ziehl-Neelson Procedure

Carbolfuchsin (Primary Stain)

- Basic fuchsin . . . . . 0.3 g
- Ethanol . . . . . 10.0 mL
- Distilled or deionized water . . . . . 95.0 mL
- Phenol . . . . . 5.0 mL

1 Dissolve the basic fuchsin in the ethanol.

2 Dissolve the phenol in the water

3 Combine the solutions and let stand for a few days.

4 Filter before use.

Decolorizer

- Ethanol . . . . . 97.0 mL
- HCl (concentrated) . . . . . 3.0 mL

Methylene Blue (Counterstain)

- Methylene blue chloride . . . . . 0.3 g
- Distilled or deionized water . . . . . 100.0 mL

Azotobacter Cyst Stain

- Glacial acetic acid . . . . . 8.5 mL
- Sodium sulfate . . . . . 3.25 g
- Neutral red . . . . . 200.0 mg
- Light green . . . . . 200.0 mg
- Ethanol . . . . . 50.0 mL
- Distilled or deionized water . . . . . 100.0 mL

Basic Fuchsin Stain (0.5%)

- Basic fuchsin ..... 0.5 g
- dH_2O ..... 99.5 mL

Capsule Stain Procedure

Congo Red Stain (Primary Stain)

- Congo red dye ..... 5.0 g
- Distilled or deionized water ..... 100.0 mL

Maneval's Stain (Counterstain)

- Phenol (5% aqueous solution) ..... 30.0 mL
- Acetic acid, glacial ..... 10.0 mL (20% aqueous solution)
- Ferric chloride ..... 4.0 mL (30% aqueous solution)
- Basic fuchsin (1% aqueous solution) ..... 2.0 mL

Carbolfuchsin (See Acid Fast, Kinyoun Procedure)

Congo Red Stain (Capsule Stain Procedure)

Crystal Violet (See Gram Stain Procedure)

Direct Count: Staining/Diluting Agents

Agent A

- 100% saturated crystal violet-ethanol solution ..... 1.0 mL
- Ethanol ..... 40.0 mL
- NaCl ..... 0.9 g
- Distilled or deionized water ..... ≅58.1 mL

1. Dissolve the crystal violet in ethanol and filter.
2. Mix 0.9 g NaCl in approximately 55 mL of distilled or deionized water.
3. Add the Ethanol and crystal violet solutions.
4. Add water to bring the total volume up to 100 mL.

H

Agent B

- Ethanol ..... 40.0 mL
- NaCl ..... 0.9 g
- Distilled or deionized water ..... ≅60.0 mL

1. Dissolve 0.9 g NaCl in approximately 55 mL of distilled or deionized water.
2. Add the mixture to 40 mL of ethanol.
3. Add water to bring the total volume up to 100 mL.

Flagella Stain Procedure (Ryu Method)

Solution I (Mordant)

- 5% Phenol (aqueous) ..... 10.0 mL
- Tannic acid ..... 2.0 g
- Aluminum potassium sulfate × 12 H_2O ..... 10.0 mL (saturated aqueous solution)

Solution II (Stain)

- Crystal violet (saturated ethanolic solution—12 g/100 mL 95% ethanol) 1.0 mL

1. Mix 10 parts of solution I with one part solution II.
2. Filter through Whatman No. 4 filter paper.
3. Keep the final stain at room temperature in a syringe fitted with a 0.22 µm pore-size membrane filter. No need to use a needle.
4. Cap the syringe to prevent drying of the stain. This preparation will keep for several weeks.

Gram Stain Procedure

Gram Crystal Violet (Modified Hucker's) (Primary Stain)

Solution A

- Crystal violet dye (90%) ..... 2.0 g
- Ethanol (95%) ..... 20.0 mL

Solution B

- Ammonium oxalate ..... 0.8 g
- Distilled or deionized water ..... 80.0 mL

1. Combine solutions A and B. Store for 24 hours.
2. Filter before use.

Gram Decolorizer

- Ethanol (95%)
- Can use 75% ethanol and 25% acetone, but decolorization time must be reduced

Gram Iodine (Mordant)

- Potassium iodide ..... 2.0 g
- Iodine crystals ..... 1.0 g
- Distilled or deionized water ..... 300.0 mL

1. Dissolve the potassium iodide in the water *first*.
2. Dissolve the iodine crystals in the solution.
3. Store in an amber bottle.

Gram Safranin (Counterstain)

- Safranin O ..... 0.25 g
- Ethanol (95%) ..... 10.0 mL
- Distilled or deionized water ..... 100.0 mL

1. Dissolve the safranin O in the ethanol.
2. Add the water.

Methylene Blue Stain (See Acid Fast, Z-N Procedure)

Nigrosin Stain

- Nigrosin ..... 10.0 g
- Distilled or deionized water ..... 100.0 mL

Rose Bengal Stain (1%)

- Rose bengal . . . . . 0.1 g
- Distilled or deionized water . . . . . 9.9 mL

Safranin Stain (See Gram Stain)

Spore Stain Procedure

Malachite Green (Primary Stain)

- Malachite green dye . . . . . 5.0 g
- Distilled or deionized water . . . . . 100.0 mL

References

Baron, Ellen Jo, Lance R. Peterson, and Sydney M. Finegold. *Bailey & Scott's Diagnostic Microbiology,* 9th ed. St. Louis, MO: Mosby Year Book, 1994.

Eisenstadt, Bruce, C. Carlton, and Barbara J. Brown. *Methods for General and Molecular Bacteriology.* Philipp Gerhardt, R. G. E. Murray, Willis A. Wood, and Noel R. Krieg, eds. Washington, DC: American Society for Microbiology, 1994.

Farmer, J. J. III. Chap. 41 in *Manual of Clinical Microbiology,* 8th ed. Patrick R. Murray, Ellen Jo Baron, James H. Jorgensen, Michael A. Pfaller, and Robert H. Yolken, eds. Washington, DC: ASM Press, 2003.

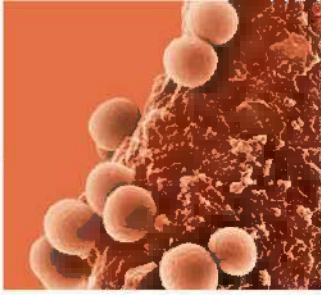
Forbes, Betty A., Daniel F. Sahm, and Alice S. Weissfeld. Chap. 19 in *Bailey & Scott's Diagnostic Microbiology,* 11th ed. St. Louis, MO: Mosby, 2002.

Koneman, Elmer W., et al. *Color Atlas and Textbook of Diagnostic Microbiology,* 5th ed. Philadelphia: Lippincott-Raven Publishers, 1997.

MacFaddin, Jean F. *Biochemical Tests for Identification of Medical Bacteria,* 2nd ed. Baltimore: Lippincott Williams & Wilkins, 1980.

Zimbro, Mary Jo and David A. Power, eds. *Difco™* and BBL™ Manual—Manual of Microbiological Culture Media. Sparks, MD: Becton Dickinson and Co., 2003.

GLOSSARY



A

absorbance A measurement, using a spectrophotometer, of how much light entering a substance is *not* transmitted.

acetoin A four-carbon intermediate in the conversion of pyruvic acid to 2,3-Butanediol; the compound detected in the Voges-Proskauer test.

acetyl-CoA Compound that enters the Krebs cycle by combining with oxaloacetate to form citrate; may be produced from pyruvate in carbohydrate metabolism or from oxidation of fatty acids.

acid clot Pink clot formed in litmus milk from the precipitation of casein under acidic conditions; an indication of lactose fermentation.

acidic stain Staining solution with a negatively charged chromophore.

acidophil *See* eosinophil.

acidophile Microorganism adapted to a habitat below pH 5.5.

acid reaction Pink color reaction in litmus milk from acidic conditions produced by lactose fermentation.

adenosine triphosphate (ATP) In hydrolysis of ATP to adenosine diphosphate (ADP), supplies the energy necessary to perform most work in the cell.

aerobe Microorganism that requires oxygen for growth.

aerobic respiration A type of energy releasing metabolism in which oxygen is the final electron acceptor in the pathway. It also has the highest potential ATP yield of all catabolic and exergonic reactions.

aerosol Droplet nuclei that remain suspended in the air for a long time.

aerotolerance Designation of an organism based on its ability to grow in the presence of oxygen.

aerotolerant anaerobe Anaerobe that grows in the presence of oxygen but does not use it metabolically.

agar overlay Used in plaque assays, the soft agar containing a mixture of bacteriophage and host poured over nutrient agar as an inoculum.

agglutination Visible clumping produced when antibodies and particulate antigens react.

agranulocyte Category of white blood cells characterized by the absence of prominent cytoplasmic granules; includes monocytes and lymphocytes.

akinete A thick-walled resting spore produced by some cyanobacteria. It is derived from a vegetative cell.

alkaliphile Microorganism adapted to a habitat above pH 8.5.

alpha (α) hemolysis The greening around a colony on blood agar as a result of partial destruction of red blood cells; typical of certain members of *Streptococcus*.

ammonification The metabolic production of ammonia. *See* deamination.

amphitrichous Describes a flagellar arrangement with flagella at both ends of an elongated cell.

amylase Family of enzymes that hydrolyze starch.

anaerobe Microorganism that cannot tolerate oxygen.

anaerobic respiration A type of energy releasing metabolism in which an inorganic compound other than oxygen is the final electron acceptor. These include NO_3, NO_2, SO_4, and CO_2. The ATP yield is higher than fermentation, but not as high as aerobic respiration.

annealing The attachment of a primer to a complimentary sequence of DNA. Annealing is done on each strand of the denatured DNA in positions flanking the desired sequence (template) to be replicated. Annealing is the second of three phases in a PCR cycle, following denaturation and prior to extension.

antibiotic Refers to an antimicrobial substance produced by a microorganism such as a bacterium or fungus.

antibody A glycoprotein produced by plasma cells, in response to an antigen, that reacts with the antigen specifically.

antigen A molecule of high molecular weight and complex three-dimensional shape that stimulates the production of antibodies and reacts specifically with them.

antimicrobic(al) Describes any substance that kills microorganisms, natural or synthetic.

antiporter A system that simultaneously transports substances in opposite directions across the cytoplasmic membrane.

antiserum General term applied to a serum that contains antibodies of a specific type.

Ascomycetes A division of fungi that produces sexual spores in a saclike structure called an ascus.

aseptic Describes the condition of being without contamination.

assimilation (nitrogen) The incorporation of NH_4 or NO_3 into organic form (amino acids) by plants.

assimiliatory sulfate reduction The conversion of sulfate (SO_4^{2-}) to a sulfhydryl group (–SH) in the biosynthesis of amino acids cysteine and methionine.

autoinducer Substance secreted by bioluminescent bacteria that, upon reaching sufficient concentration, triggers the bioluminescent reaction; believed to help conserve energy by synchronizing individual cellular reactions.

autotroph An organism that is able to make all of its organic molecules using CO_2 as the source of carbon. *See also* heterotroph.

auxotroph A nutritional mutant; a cell incapable of synthesizing the nutrient that is acting as the marker in a specific genetic experiment; will grow only on a complete medium. *See also* prototroph.

B

bacillus Rod-shaped cell.

back mutation Mutation in a gene that reverses the effect of an original mutation.

bacteriophage Virus that attacks bacteria; usually host-specific.

basic stain Staining solution with a positively charged chromophore.

Basidiomycetes Division of fungi characterized by club-like appendages (basidia) that produce haploid spores; include common mushrooms and rusts.

basophil Category of white blood cells; one of three types of granulocyte (along with eosinophils and neutrophils) characterized by a cytoplasm with dark-staining granules and a lobed nucleus (often difficult to see because of the dark cytoplasmic granules).

β-hemolysis The clearing around a colony on blood agar resulting from complete destruction of red blood cells; typical of certain members of *Streptococcus*.

β-lactamase Enzyme found in some bacteria that breaks a bond in the β-lactam ring of β-lactam antibiotics, rendering the antibiotic ineffective; the source of resistance against these antibiotics.

β-lactam antibiotic A group of structurally related antimicrobial chemicals that interfere with cross-linking of peptidoglycan subunits, which results in a defective wall structure and cell lysis; examples are penicillins and cephalosporins.

biofilm A layer of bacterial cells adhering to and reproducing on a surface; used commercially for acetic acid production and water purification.

bioluminescence Process by which a living organism emits light.

biosafety level (BSL) One of four sets of minimum standards for laboratory practices, facilities, and equipment to be used when handling organisms at each level; BSL-1 requires the least care, BSL-4 the most.

bound coagulase An enzyme (also called clumping factor) bound to the bacterial cell wall, responsible for causing the precipitation of fibrinogen and coagulation of bacterial cells.

C

capnophile Microaerophile that requires elevated CO_2 levels.

capsule Insoluble, mucoid, extracellular material surrounding some bacteria.

carbohydrate One of four families of biochemicals; characterized by containing carbon, hydrogen, and oxygen in the ratio 1:2:1.

carcinogen Any substance that causes cancer.

cardinal temperatures Minimum, optimum, and maximum temperatures for an organism.

casease A family of hydrolytic enzymes that break down casein, often extracellularly.

casein Milk protein that gives milk its white color.

catalase Enzyme produced by some bacteria that catalyzes the breakdown of metabolic H_2O_2.

cestode The class of parasitic worms commonly called tapeworms; characterized by having a head (scolex) with suckers and hooks and numerous segments (proglottids) that are little more than sex organs.

chemoheterotroph Any organism that uses chemicals for energy and organic compounds as a carbon source. Usually the same organic compounds serve both purposes.

chemolithotroph Any organism that gets its energy from chemicals and its electrons from an inorganic source. No eukaryotes are chemolithotrophic, only some prokaryotes such as sulfur and ammonia oxidizing bacteria.

chromogenic reducing agent A substance that produces color when it gives up electrons (becomes oxidized).

chromophore The charged region of a dye molecule that gives it its color.

coagulase *See* bound coagulase; free coagulase.

coagulase-reacting factor Plasma component that reacts with free coagulase to trigger the clotting mechanism.

coccus Spherical cell.

coenzyme Nonprotein portion of an enzyme that aids the catalytic reaction (usually by accepting or donating electrons).

coliform Member of *Enterobacteriaceae* that ferments lactose with production of gas within 48 hours at 37°C.

colony Visible mass of cells produced on culture media from a single cell or single CFU (cell forming unit); a pure culture.

colony forming unit Term used to define the cell or group of cells (e.g., staphylococci, streptococci) that produces a colony when transferred to plated media.

commensal Describes a synergistic relationship between two organisms in which one benefits from the relationship and the other is affected neither negatively nor positively.

common source epidemic An epidemic in which the disease is transmitted from a single source (such as water supply) and is not transmitted person-to-person.

compatible solutes Compounds such as amino acids that function both as metabolic constituents and solutes necessary to maintain osmotic balance between the internal and external environments.

competent cell Cell capable of picking up DNA from the environment; some cells are naturally competent, in other cases the cells are made to be competent artificially.

competitive inhibition Process whereby a substance attaches to an enzyme's active site, thereby preventing attachment of the normal substrate.

complete medium A medium used in genetic experiments that supplies all nutrients for growth required by prototrophs and auxotrophs.

complex medium A medium in which at least one nutritional ingredient is of unknown composition or amount. Media with any plant or animal extract are always complex.

confirmatory test One last test after identification of an unknown, to further support the identification.

conjugate acid A compound (one of a conjugate pair) that donates a proton in solution.

conjugate base A compound (one of a conjugate pair) that accepts a proton in solution.

conjugate pair A compound that alternates between acid and base forms by losing or gaining one proton. Example: HCl/Cl^-.

constitutive Term used to describe an enzyme that is produced continuously, as opposed to an enzyme produced only when its substrate is present. *See also* induction.

cos site (cohesive end sites) In Lambda phage (linear) DNA, the single stranded tails at the 5' ends of both strands are complimentary to each other and can form a circular molecule. These "sticky" ends can be a source of error in DNA fragment size determinations if not properly heated before running gel electrophoresis.

counterstain Stain applied after decolorization to provide contrast between cells that were decolorized and those that weren't.

culture A liquid or solid medium with microorganisms growing in or on it.

cyst "Resting stage" in life cycle of certain protozoans. *See also* trophozoite.

cytochrome A class of iron-containing enzyme in electron transport chains; characterized by a porphyrin ring structure.

cytochrome oxidase An electron carrier in the electron transport chain of aerobes, facultative anaerobes, and microaerophiles that makes the final transfer of electrons to oxygen.

cytoplasm The semifluid component of cells inside the cellular membrane in which many chemical reactions take place.

D

deaminase Enzyme that catalyzes the removal of the amine group (NH_2) from an amino acid.

death phase Closed system microbial growth phase immediately following stationary phase; characterized by population decline usually resulting from nutrient deficiencies or accumulated toxins.

decarboxylase Enzyme that catalyzes the removal of the carboxyl group (COOH) from an amino acid.

decimal reduction value Amount of time at a specific temperature to reduce a microbial population by 90% (one log cycle).

defined medium A growth medium in which the exact amount and chemical formula (and thus identity) of each ingredient are known.

denaturation The first of three phases in a PCR cycle, followed by annealing and extension. The separation of the strands in double stranded DNA.

denitrification The bacterial process in which nitrate is reduced to various forms of nitrogen in its role as final electron acceptor of anaerobic respiration.

deoxyribonuclease A family of hydrolytic enzymes that depolymerize DNA into polynucleotides. The precise location of hydrolysis within the sugar-phosphate backbone depends on which DNase is acting. These are usually secreted enzyme.

Deuteromycetes An unnatural grouping of fungi in which the sexual stages are either unknown or are not used in classification.

differential medium Growth medium that contains an indicator (usually color) to detect the presence or absence of a specific metabolic activity.

differential stain Staining procedure that allows distinction between cell types or parts of cells; often involves more than one stain, but not necessarily.

dilution blank A test tube containing a measured volume of sterile diluent (water, saline, or buffer) used to serially dilute a concentrated solution or broth.

dilution factor Proportion of original sample present in a new mixture after it has been diluted; calculated by dividing the volume of the original sample by the total volume of the new mixture. (Subsequent dilutions in a serial dilution must be multiplied by the dilution factor of the previous dilution.)

diploid Defines a cell that has two complete sets of genetic information; one stage in the life cycle of all eukaryotic microorganisms (which also have a haploid [one set] stage).

direct agglutination Serological test in which the combination of antibodies and *naturally* particulate antigens, if positive, form a visible aggregate. *See also* indirect agglutination.

disaccharide A sugar made of two monosaccharide subunits; e.g., the monosaccharides glucose and galactose can combine to make the disaccharide lactose.

disk diffusion test (Kirby-Bauer Test) This is one way to check susceptibility and resistance of a microbe to an antibiotic. A paper disk containing an antibiotic is placed on a plate inoculated to produce confluent growth of a bacterium. Susceptibility is determined by the size of the zone of inhibition of growth around the disk.

disproportionation reaction A redox reaction of some sulfur reducers whereby a substrate is split into two molecules, one that is more reduced and one that is more oxidized than the original compound. Example: $S_2O_3^{2-} \longrightarrow SO_4^{2-} + H_2S$.

dissimilatory sulfate reduction A respiratory reaction in which sulfate is reduced exclusively for the purpose of gaining energy.

DNA polymerase A group of enzymes that catalyze the addition of deoxyribonucleotides to the 3' end of an existing polynucleotide chain.

DNase *See* deoxyribonuclease.

Durham tube A small, inverted test tube used in some liquid media to trap gas bubbles and indicate gas production.

E

electromagnetic energy The energy that exists in the form of waves (including x-rays, ultraviolet, visible light, and radio waves).

electron donor A compound that can be oxidized to transfer electrons to another compound, which in turn becomes reduced.

electron transport chain A series of membrane-bound electron carriers that participate in oxidation-reduction reactions whereby electrons are transferred from one carrier to another until given to the final electron acceptor (oxygen in aerobes, other inorganic substances in anaerobes) in respiration.

endergonic Any metabolic reaction in which the products have more potential energy than the reactants. These are usually associated with anabolic (synthesis) reactions.

endospore Dormant, highly resistant form of bacterium; produced only by species of *Bacillus*, *Clostridium*, and a few others.

enteric bacteria Informal name given to bacteria that occupy the intestinal tract.

Enterobacteriaceae A group of Gram-negative rods that also are oxidase-negative, ferment glucose to acid, have polar flagella if motile, and usually are catalase-positive and reduce nitrate to nitrite.

enzyme A protein that catalyzes a metabolic reaction by interacting with the reactant(s) specifically; each metabolic reaction has its own enzyme.

eosinophil A category of white blood cells; one of three types of granulocyte (along with basophils and neutrophils) characterized by a cytoplasm with red (in typical stains) granules and lobed nucleus.

epitope That portion of an antigen that stimulates the immune system and reacts with antibodies.

eukaryote Type of cell with membranous organelles, including a nucleus, and having 80S ribosomes and many linear molecules of DNA.

exergonic Any metabolic reaction in which the reactants have more potential energy than the products. These are usually associated with catabolic (degradative) reactions.

exoenzyme Enzyme that operates in the external environment after being secreted from the cells.

exponential phase Closed-system microbial growth phase immediately following the lag phase; characterized by constant maximal growth during which population size increases logarithmically.

extension The third of three phases in PCR, following denaturation and annealing, whereby a polymerase (usually *Taq*I) catalyzes the replication of a DNA template.

extracellular Pertains to the region outside a cell.

extreme halophile Organism that grows best at 15% or higher salinity.

extreme thermophile Organism that grows best at temperatures above 80°C.

F

facultative anaerobe Microorganism capable of both fermentation and respiration; grows in the presence or absence of oxygen.

facultative thermophile Microorganism that prefers temperatures above 40°C but will grow in lower temperatures.

false negative Test result that is negative when the sample is actually positive; usually a result of lack of sensitivity in the test system.

false positive Test result that is positive when the sample is actually negative; usually a result of lack of specificity of the test system.

fastidious (microorganism) Describes a microorganism with strict physiological requirements; difficult or impossible to grow unless specific conditions are provided.

fatty acid A long chain, organic molecule with a carboxylic acid at one end with the rest of the carbons being bonded to hydrogens.

fermentation Metabolic process in which an organic molecule acts as an electron donor and one or more of its organic products act as the final electron acceptor, marking the end of the metabolic sequence (differs from respiration in that an inorganic substance is not needed to act as final electron acceptor).

final electron acceptor (FEA) The molecule receiving electrons (it becomes reduced) at the end of a metabolic sequence of oxidation/reduction reactions.

flavin adenine dinucleotide (FAD) Coenzyme used in oxidation/reduction reactions that acts as an electron acceptor or donor, respectively.

flavoprotein A flavin-containing protein in the electron transport chain, capable of receiving and transferring electrons as well as entire hydrogen atoms; sometimes bypasses normal route of transfer and reduces oxygen directly, forming hydrogen peroxide and superoxide radicals.

free coagulase An enzyme produced and secreted by some microorganisms that initiates the clotting mechanism in plasma; seen as a solid mass in the coagulase tube test or clumps in the slide test.

free energy (of formation) The energy released by a molecule in a chemical reaction that is able to do work (e.g., produce ATP). It is equivalent to the energy required to form the molecule from its individual elements.

free living A term used to describe nonparasitic organisms.

G

gametangium A structure that produces gametes; used in describing fungi.

gamete Reproductive cell that must undergo fusion with another gamete to continue the life cycle; usually are haploid. *See also* spore.

gelatinase Enzyme secreted by microorganisms, which catalyzes the hydrolysis of gelatin.

generation time The time required for a population to produce offspring (i.e., in bacteria that reproduce by binary fission, the time needed for the population to double); inverse of mean growth rate.

germ theory Theory holding that diseases and infections are caused by microorganisms; first hypothesized in the 16th century by Girolamo Fracastoro of Verona.

glycolysis Metabolic process by which a glucose molecule is split into two 3-carbon pyruvic acid molecules, producing two ATP (net) and two $NADH_2$ molecules.

gradient bacteria Microaerophiles with very narrow nutritional requirements living in habitats characterized by nutrients diffusing upward from sediments below and oxygen diffusing downward from air above.

granulocyte A category of white blood cells characterized by prominent cytoplasmic granules; includes neutrophils, eosinophils, and basophils.

group A streptococci α-hemolytic members of the genus *Streptococcus*, characterized by possessing the Lancefield group A antigen; belong to the species *S. pyogenes*, an important human pathogen.

group B streptococci β-hemolytic members of the genus *Streptococcus*, characterized by possessing the Lancefield group B antigen; belong to the species *S. agalactiae*.

group D streptococci Streptococci possessing the Lancefield group D antigen; include *S. bovis* and species of the genus *Enterococcus*.

H

halophile Microorganism that grows best at 3% or higher salinity.

haploid Describes a cell that has one complete set of genetic information; all eukaryotic microorganisms have a haploid stage and a diploid (two sets) stage in their life cycle; all prokaryotes are haploid.

hemagglutination General term applied to any agglutination test in which clumping of red blood cells indicates a positive reaction.

hemoglobin Iron-containing protein of red blood cells that is responsible for binding oxygen.

hemolysin A class of chemicals produced by some bacteria that break down hemoglobin and produce hemolysis.

heterocyst This is a hollow-appearing cell found in some cyanobacteria specialized to perform nitrogen fixation.

heterotroph An organism that requires carbon in the form of organic molecules. *See also* autotroph.

host An organism that serves as a habitat for another organism such as a parasite or a commensal.

hydrolysis The metabolic process of splitting a molecule into two parts, adding a hydrogen ion to one part and a hydroxyl ion to the other. (One water molecule is used in the process.)

hyperosmotic (solution) A term used to describe extracellular solute concentration relative to the cell; a solution that contains a higher concentration of solutes than the cell, such that water tends to move down its concentration gradient and diffuse out of the cell.

hypha (*pl.* hyphae) A filament of fungal cells.

hyposmotic A term used to describe extracellular solute concentration relative to the cell; a solution that contains a lower concentration of solutes than the cell, such that water tends to move down its concentration gradient and diffuse into the cell.

I

IMViC Acronym representing the four tests used in identification of *Enterobacteriaceae*: Indole, Methyl Red, Voges-Proskauer, and Citrate.

incomplete medium *See* minimal medium.

incubation The process of growing a culture by supplying it with the necessary environmental conditions.

index case First occurrence of an infection or disease that results in an epidemic.

indirect agglutination Serological test in which artificially produced particulate antigens or antibodies are used to form a visible aggregate if positive. *See also* direct agglutination.

induction Process by which a substrate (inducer) causes the transcription of the genes used in its digestion.

infectious disease A transmissible illness or infection.

inoculum The organisms used to start a new culture or transferred to a new place.

inorganic molecule A molecule that does *not* contain carbon and hydrogen.

intracellular Within the cell; as an intracellular enzyme catalyzing reactions inside the cell.

iron-sulfur protein Iron-containing electron carrier in electron transport chains. Differentiated from cytochromes by the lack of porphyrin ring structure.

isolate (*v.*) The process of separating individual cell types from a mixed culture; (*n.*) the group of cells resulting from isolation.

isosmotic Describe extracellular solute concentration relative to the cell; a solution that contains the same concentration of solutes as the cell, such that water tends to move equally into and out of the cell.

K

karyogamy The process of nuclear fusion that occurs after fertilization (plasmogamy) in sexual life cycles.

Krebs cycle A cyclic metabolic pathway found in organisms that respire aerobically or anaerobically.

L

lag phase Closed-system microbial growth phase immediately preceding the exponential phase; characterized by a period of adjustment in which no growth takes place.

limit of resolution The closest two points can be together for the microscope lens to make them appear separate; two points closer than the limit of resolution will blur together.

ligate To join together.

lipase A family of hydrolytic enzymes that break down fats into their component parts: glycerol and up to three fatty acids.

lipid A fat.

lophotrichous Describes a flagellar arrangement with a group of flagella at one end of an elongated cell.

lymphocyte A category of white blood cells; one of two types of agranulocyte (along with monocytes); characterized by a large nucleus and little visible cytoplasm; involved in specific acquired immunity as T-cells and B-cells.

lysozyme A naturally occurring bactericidal enzyme in saliva, tears, urine, and other body fluids; functions by breaking peptidoglycan bonds.

lytic cycle The viral life cycle from attachment to lysis of the host cell.

M

mean growth rate constant The number of generations produced per unit time; the inverse of generation time.

medium A substance used for growing microbes; may be liquid (usually a broth) or solid (usually agar).

meiosis The process in which the nucleus of a diploid eukaryotic cell nucleus divides to make four haploid nuclei.

mesophile A microorganism that grows best at temperatures between 15°C and 45°C.

methylase An enzyme that functions to add methyl groups ($-CH_3$) to adenine or cytosine bases within the DNA recognition sequence, which is thus modified and protected from the endonuclease.

methylation A protective enzymatic mechanism in many organisms whereby methyl groups ($-CH_3$) attach to DNA, thus blocking attachment and destruction by restriction endonucleases.

microaerophile A microorganism that requires oxygen, but can't tolerate atmospheric levels of it.

microbial growth curve Graphic representation of microbial growth in a closed system, consisting of lag phase, exponential (log) phase, stationary phase, and death phase.

minimal medium A medium used in genetic experiments, supplying all nutrients for growth *except* the one required by auxotrophs, and thus supporting growth of only prototrophs.

minimum inhibitory concentration The lowest concentration of an antimicrobial substance required to inhibit growth of all microbial cells it contacts; on an agar plate, typically the outer edge of the zone of inhibition where the substance has diffused to the degree that it no longer inhibits growth.

mitosis The process in which a nucleus divides to produce two identical nuclei.

mixed acid fermentation Vigorous fermentation producing many acid products including lactic acid, acetic acid, succinic acid, and formic acid, and subsequently lowering the pH of the medium to pH 4.4 or below.

mold Informal grouping of filamentous fungi. *See also* yeast.

monocyte A category of white blood cells; one of two types of agranulocyte (along with lymphocytes); characterized by large size and lack of cytoplasmic granules; the blood form of macrophages.

monotrichous Describes a flagellar arrangement consisting of a single flagellum.

morbidity Epidemiological measurement of incidence of a disease; typically accompanied by "incidence rate," referring to the incidence of a disease over time.

morphology The shape of an organism.

mortality Epidemiological measurement of death caused by a disease; typically accompanied by "incidence rate," referring to the incidence of death from a disease over time.

mutagen A substance that causes mutation in DNA; most mutagens are carcinogens.

mutation Alteration in a cell's DNA.

mutualistic Describes a synergistic relationship between two organisms in which both benefit from the interaction.

mycelium A mass of fungal filaments (hyphae).

N

nematode A class of roundworms; environmentally abundant in some parasitic species.

neutrophil Category of white blood cells; one of three types of granulocyte (along with eosinophils and basophils) characterized by a granular cytoplasm and lobed nucleus; also known as "polymorphonuclear granulocytes" or "PMNs."

neutrophile Microorganism adapted to a habitat between pH 5.5 and 8.5.

nicotinamide adenine dinucleotide (NAD) A coenzyme used in oxidation/reduction reactions that acts as an electron acceptor or donor, respectively.

nisin Antibiotic produced by *Lactococcus lactis*.

nitrate A highly oxidized form of nitrogen; NO_3.

nitrate reductase An enzyme produced by all members of *Enterobacteriaceae* (and others) that catalyzes the reduction of nitrate (NO_3) to nitrite (NO_2).

nitrification The chemical process in which nitrogen compounds, such as ammonia and nitrite are oxidized to form nitrate. Typically, different bacteria perform these two steps in which ammonia is first oxidized to nitrite, which is subsequently oxidized to nitrate.

nitrite NO_2, an oxidized form of nitrogen.

nitrogen fixation The bacterial process in which gaseous nitrogen (N_2) becomes reduced to NH_4. This is ecologically important because N_2, while abundant in the atmosphere, is not in a form usable by most organisms, whereas NH_4 is usable by plants and can thus enter food chains.

nitrogenase The enzyme involved in nitrogen fixation, that is, converting gaseous N_2 to NH_4. This is performed by free-living bacteria (e.g., *Azotobacter*), symbiotic bacteria (e.g., *Rhizobium*), and many cyanobacteria.

noninfectious diseases Conditions not caused by microorganisms; examples are stroke, heart disease, and emphysema.

O

objective lens The microscope lens that first produces magnification of the specimen in a compound microscope.

obligate (strict) aerobe Microorganism that requires oxygen to survive and grow.

obligate (strict) anaerobe Microorganism for which oxygen is lethal; requires the complete absence of oxygen.

obligate thermophile Microorganism that grows only at temperatures above 40°C.

ocular lens The lens the microscopist looks through; produces the virtual image by magnifying the real image.

ocular micrometer A uniformly graduated linear scale placed in the microscope ocular used for measuring microscopic specimens.

oligonucleotide A short nucleic acid molecule.

operon A prokaryotic structural and functional genetic unit consisting of two or more structural genes that code for enzymes in the same pathway and that are regulated together.

opportunistic pathogen A microorganism not ordinarily thought of as pathogenic (i.e., most enterics) that will cause infection when out of its normal habitat.

organic molecule A molecule made of reduced carbon—that is, containing at least carbon and hydrogen.

osmosis Diffusion of water across a semipermeable membrane.

osmotolerant Microorganism that will grow outside of its preferred salinity range.

oxidation/reduction Chemical reaction in which electrons are transferred. (The molecule losing the electrons becomes oxidized; the molecule gaining the electrons becomes reduced.)

oxidative deamination The metabolic process in which an amino acid has its amine removed, producing ammonia and an organic acid.

oxidative phosphorylation The process by which an electron transport chain is used to add a phosphate to ADP to make ATP.

oxidizing agent A substance that removes electrons from (oxidizes) another. *See also* reducing agent.

P

palindrome A word, phrase, number, or other sequence of characters that reads the same backward or forward.

parasite An organism that lives symbiotically with another, but to the detriment of the other organism (called a host).

parthenogenesis A process in which females produce offspring from an unfertilized egg.

peptidoglycan The insoluble, porous, cross-linked polymer comprising bacterial cell walls; generally thick in Gram-positive organisms and thin in Gram-negative organisms.

peptone A digest of protein used in formulating some bacteriological media.

peritrichous Describes a flagellar arrangement in which flagella arise from the entire surface of the cell.

pH The measure of a solution's alkalinity or acidity; the negative logarithm of the hydrogen ion concentration.

phage *See* bacteriophage.

phage host Bacteria attacked by a virus.

phototaxis Phototaxis is a response to light stimuli. Positive phototaxis is movement toward a light stimulus, whereas negative phototaxis is movement away from light.

plaque The clearing produced in a bacterial lawn as a result of cell lysis by a bacteriophage; used to calculate phage titer (PFU/mL) when accompanied by a serial dilution.

plaque forming unit (PFU) Term that replaces "viral particle" or "single virus" when referring to phage titer; accounts for multiple particle arrangements in which more than one virus is responsible for initiating a plaque.

plasma The noncellular (fluid) portion of blood; consists of serum (including serum proteins) and clotting proteins.

plasmid Small, circular, extrachromosomal piece of DNA found in prokaryotic cells; often carries genes for antibiotic resistance.

plasmogamy The cytoplasmic fusion of gametes at the time of fertilization. *See also* karyogamy.

plasmolysis Shrinking of cell membrane (pulling away from the rigid cell wall) because of loss of water to the environment and reduced turgor pressure.

polar flagellum A single flagellum at one end of an elongated cell.

pour plate technique Method of plating bacteria in which the inoculum is added to the molten agar prior to pouring the plate.

preadsorption period When performing a plaque assay, the time given to allow a bacteriophage to attach to the host before adding the mixture to the molten agar being plated.

precipitate An insoluble material that comes out of a solution during a precipitation reaction.

presumptive identification Tentative identification of an isolate based on one or more key test results.

primary stain The first stain applied in many differential staining techniques; usually subjected to a decolorization step that forms the basis for the differential stain.

primer A short nucleotide sequence (typically 10–20 nucleotides) used in PCR to attach to DNA for the purpose of replicating a desired sequence of nucleotides. Typically, two primers attach to sites on opposite strands, flanking the area (template) to be replicated.

proglottid Tapeworm segments posterior to the scolex, used for absorption of nutrients and containing reproductive organs.

prokaryote Type of cell lacking internal compartmentalization (membranous organelles, including a nucleus) and having 70S ribosomes and a circular molecule of DNA; more primitive than eukaryotes.

promoter site The patch of DNA upstream from the structural gene(s), which binds RNA polymerase to begin transcription.

propagated transmission Conveying a disease person-to-person.

proteolytic Refers to catabolism of protein; e.g., a proteolytic enzyme.

prototroph A strain that is capable of synthesizing the nutrient that is acting as the marker in a particular genetic experiment; prototrophs will grow on complete and minimal medium. *See also* auxotroph.

pseudohypha A chain of fungal cells produced by budding (rather than by cytokinesis that produces two equally sized cells) and which is characterized by constrictions at cell junctions.

psychrophile A microorganism that grows only at temperatures below 20°C, with an optimum around 15°C.

psychrotroph A microorganism that grows optimally at temperatures between 20 and 30°C but will grow at temperatures as low as 0°C and as high as 35°C.

pure culture Microbial culture containing only a single species.

purine nucleotide Purines are made of a sugar (ribose or deoxyribose), phosphate, and a nitrogen-containing base composed of two joined rings. Examples are adenine (A) and guanine (G). These are found in DNA and RNA.

putrefaction The process of digesting dead organic material; decay.

pyrimidine nucleotide Pyrimidines are made of a sugar (ribose or deoxyribose), phosphate, and a nitrogen-containing base composed of a single ring. Examples are cytosine (C) in DNA and RNA, thymine (T) in DNA, and uracil (U) in RNA.

pyruvic acid (pyruvate) A three-carbon compound produced at the end of glycolysis that may enter a respiration or a fermentation pathway; also serves as a starting point for synthesis of certain amino acids and an entry point for their digestion.

Q

quinoidal (compound) A color-producing compound containing quinone as its central structure.

quorum sensing The phenomenon in bioluminescing bacteria whereby the light-emitting reaction of all cells takes place simultaneously when a threshold concentration of secreted autoinducer is reached.

R

real image Magnified image of a specimen produced by the objective lens of a microscope; the real image is magnified again by the ocular lens to produce the virtual image.

recognition site A short DNA sequence where a restriction enzyme attaches. Each enzyme recognizes and attaches to its own specific sequence.

reducing agent A substance that donates electrons to (reduces) another. *See also* oxidizing agent.

reductase An enzyme that catalyzes the transfer of electrons from donor molecule to acceptor molecule, thereby reducing the acceptor.

reduction *See* oxidation/reduction.

refraction The bending of light as it passes from a medium with one refractive index into another medium with a different refractive index.

reservoir A nonhuman host or other site in nature serving as a perpetual source of pathogenic organisms.

resolution The clarity of an image produced by a lens; the ability of a lens to distinguish between two points in a specimen; high resolution in a microscope is desirable.

resolving power *See* limit of resolution.

respiration Metabolic process by which an organic molecule acts as an electron donor and an inorganic substance—such as oxygen, sulfur, or nitrate—acts as the final electron acceptor in an electron transport chain, marking the end of the metabolic sequence (differs from fermentation, which uses one of its own organic products as the final electron acceptor).

restriction enzyme Enzymes involved in cutting DNA at specific recognition sites, unique to the enzyme. Involved in removing damaged DNA and destroying foreign (e.g., phage) DNA.

reticuloendothelial system Combination of macrophages and associated cells located in the liver, spleen, bone marrow, and lymph nodes.

reverse citric acid cycle An energy consuming process used by green sulfur bacteria whereby molecules ordinarily associated with the oxidative respiratory reactions of the Krebs cycle perform reactions in the opposite direction to reduce carbon dioxide and form pyruvate.

reverse electron flow An energy consuming process used by purple sulfur bacteria whereby electrons transferred down the ETC of the photosystem are boosted upward against their thermodynamic gradient to reduce $NADP^+$ needed by the Calvin cycle.

reversion In carbohydrate fermentation tests, the phenomenon of a microorganism fermentively depleting the carbohydrate and reverting to amino acid metabolism, thereby neutralizing acid products with alkaline products; produces a false negative.

rhizoid A root-like structure used for attachment of some fungi to the substrate.

RNA polymerase A group of enzymes that catalyze the addition of ribonucleotides to the 3' end of an existing polynucleotide chain.

S

saltern Salterns are low pools of saltwater. Evaporation of the water leaves salt, which can then be harvested.

saprophyte A heterotroph that digests dead organic matter; a decomposer.

scolex The "head" of a tapeworm, often with suckers and hooks for attachment.

selective medium Growth medium that favors growth of one group of microorganisms and inhibits or prevents growth of others.

sensitivity This is a measure of a test's ability to detect small amounts of the item being tested for. The better the sensitivity, the fewer the false negatives (due to smaller amounts triggering a positive reaction), and the more useful the test is.

serial dilution Series of dilutions used to reduce the concentration of a culture and thereby produce between 30 and 300 colonies when plated, providing a means of calculating the original concentration.

serology A discipline that utilizes a serum containing antibodies (antiserum) to detect the presence of antigens in a sample; also refers to identification of antibodies in a patient's serum.

serum Fluid portion of blood minus the clotting factors.

soft agar A semisolid growth medium containing a reduced concentration of agar; used in plaque assay to allow diffusion of bacteriophage while arresting movement of the bacteriophage host.

solute The dissolved substance in a solution.

solution The mixture of dissolved substance (solute) and solvent (liquid).

solvent The liquid portion of a solution in which solute is dissolved.

specificity This is a measure of a test's ability to produce a positive response only when reacting with the particular item being tested for. The better the test's specificity, the fewer the false positives (due to better discrimination), and the more useful it is.

spirillum Spiral-shaped cell.

sporangium Structure that produces spores.

spore In bacteria, a dormant form of a microbe protected by specialized coatings produced under conditions of, and resistant to, adverse conditions; also known as an endospore; in fungi and plants, spores are specialized reproductive cells; frequently a means of dissemination.

spread plate technique Method of plating bacteria in which the inoculum is transferred to an agar plate and spread with a sterile bent-glass rod or other spreading device.

stage micrometer A microscope ruler used to calibrate an ocular micrometer.

standard curve A graph constructed from data obtained using samples of known value for the independent variable; once made, can be used to experimentally determine the value of the independent variable when the dependent variable is measured on an unknown.

stationary phase Closed system microbial growth phase immediately following exponential phase; characterized by steady, level growth during which death rate equals reproductive rate.

stolons Surface hyphae of some molds (e.g., *Rhizopus*) that attach to the substrate with rhizoids.

stormy fermentation Vigorous fermentation produced in litmus milk by some species that produce an acid clot but subsequently break it up because of heavy gas production (members of *Clostridium*).

streptolysin Hemolysin (blood hemolyzing exotoxin) produced and secreted by members of *Streptococcus*.

superoxide dismutase Enzyme produced by some bacteria that catalyzes the conversion of superoxide radicals to hydrogen peroxide.

syntrophy A situation where two or more organisms, having differing metabolic capabilities, derive mutual benefit by providing and/or receiving essential nutrients otherwise not available to them.

T

template DNA A particular sequence of DNA to be replicated in the polymerase chain reaction process.

thermal death time The amount of time required to kill a population of a specific size at a specific temperature.

thermophile A microorganism that grows best at temperatures above 40°C.

titer A measurement of concentration of a substance or particle in a solution; used in measurements of phage concentration.

transformant cells Cells that have undergone transformation by picking up foreign DNA in a genetic engineering experiment.

transformation A form of genetic recombination performed by some bacteria in which DNA is picked up from the environment and incorporated into its genome.

trematode A class of parasitic flatworms; also known as "flukes."

trend line A line drawn on a graph to show the general relationship between X and Y variables; also known as a regression line.

triacylglycerol *See* triglyceride.

tricarboxylic acid cycle *See* Krebs cycle.

trichome A filament of cyanobacterial cells. It does not include the sheath, if present.

triglyceride A molecule composed of glycerol and three long chain fatty acids.

trophozoite "Feeding" stage in the life cycle of certain protozoans. *See also* cyst.

tryptophan An amino acid.

tryptophanase Enzyme that catalyzes the hydrolysis of tryptophan into indole and pyruvic acid; detected in the indole test.

turgor pressure The pressure inside a cell that is required to maintain its shape, tonicity, and necessary biochemical functions.

2,3-butanediol fermentation The end-product of a metabolic pathway leading from pyruvate through acetoin with the associated oxidation of NADH; detected in the Voges-Proskauer test.

U

undefined medium *See* complex medium.

urease Enzyme that catalyzes the hydrolysis of urea into two ammonias and one carbon dioxide.

utilization medium A differential medium that detects the ability or inability of an organism to metabolize a specific ingredient.

V

variable A factor in a scientific experiment that is changed; the control and experimental groups in a good experiment differ in only one variable, the one being tested.

vector In genetic engineering, a means, often a plasmid or a virus, of introducing DNA into a new host.

vegetative cell An actively metabolizing cell.

virtual image The image produced when the ocular lens of a microscope magnifies the real image; appears within or below the microscope.

Voges-Proskauer test A differential test used to identify organisms that are capable of performing a 2,3-butanediol fermentation.

W

wavelength Measurement of a wave from crest to crest, usually in nanometers—as in electromagnetic energy.

whey Watery portion of milk as seen upon coagulation of casein in the production of a curd.

X

X-Y scatter plot Graph presenting the relationship between two variables.

Y

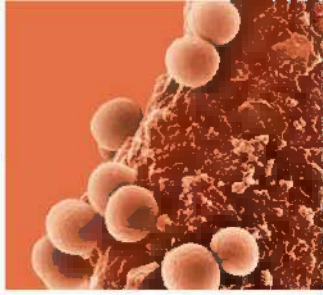
yeast An informal grouping of unicellular fungi. *See also* mold.

Z

zone of inhibition On an agar plate, the area of nongrowth surrounding a paper disk containing an antimicrobial substance. (The zone typically ends at the point where the diffusing antimicrobial substance has reached its minimum inhibitory concentration, beyond which it is ineffective.)

zygospore The product of fertilization and the site of meiosis in some molds.

zygote The product of gamete fusion (plasmogamy) and nuclear fusion (karyogamy); a fertilized egg.



INDEX

acetaldehyde, 648, 671
acetate, 671
acetyl-CoA, 287, 340, 830
acid-base reactions, 286
acid clot reaction, 412
acid-fast bacilli (AFB), 204
acid-fast stains, 195, 203–204
  Kinyoun method, 203, 204
  ZN method, 203, 205, 206
acidic stain, 191, 211
acidophiles, pH tolerance, 109
acids, weak, 286
actinobacteria, 641
actinomycetes, 204, 519
Aequorea victoria, 705
aerobic bacteria/obligate aerobes, 583, 613, 614, 621
aerobic electron transport chains. *See* electron transport chain
aerobic respiration, 287, 291, 328
Aerococcus, 424
Aeromonas sobria, 182
aerosol precaution, 31, 186
aerotolerance, 91, 321
African trypanosomiasis/sleeping sickness, 802–803
agar. *See also* milk agar, nutrient agar
  brain-heart infusion, 65
  deep stabs, 91–92
  glycerol yeast extract, 641
  m-Endo LES, 648
  Mueller-Hinton, 529
  Sabouraud, 641, 788, 789, 795
  sheep blood, 66, 67, 237, 243–244, 479
  starch, 361
  TDA, 401–402
  tryptic soy, 68
agarose gel electrophoresis, 857
  Module A, 858–859
  Module B, 859–860
  Module C, 861–862
agar plates, 23, 24, 467, 497, 529, 633, 648, 733–734, 740, 758
  inoculation with pipette, 842, 845
  inoculation with a pipettor, 845
  spot inoculation, 837
agar slants, 30, 401
  growth patterns, 75–76
  Kligler iron agar/ Triple sugar iron agar, 401
  LIA, 407
agar tubes, stab inoculation, 836
agglutination, 743, 752
  in blood typing, 765–766
  direct and indirect, 761–762
agranulocytes, 745, 746
akinetes, 169
Alcaligenes/Alcaligenes faecalis, 67, 75, 110, 295, 411
alcohols
  low molecular weight, 627
  produced by fermentation, 286
algae, green, 164, 169, 171
alkaliphiles, pH tolerance, 109
alveolata/alveolates 165, 175–176
alveolate pathogens
  Balantidium coli, 803
  Entamoeba coli, 807
  Entamoeba histolyca, 806–807
  Plasmodium spp., 803–805
  Toxoplasma gondii, 805–806
  unikont, 806
amebic dysentery, 806
American Public Health Association (APHA), 121
American Society for Microbiology, 7
Ames Test, 685, 733–735
amino acids
  aromatic, 827
  carboxyl group (COOH), 349
  catabolism, 357
  cysteine, 627
  deamination, 285, 349, 605
  decarboxylation, 349, 350
  lysine, 407
  test for, 606
aminopeptidases, 389
ammonia/ammonium, and test, 592, 595, 599, 600, 601
ammonification, 605–606
amoeba, 176
  micrographs, 143
  Unikonta, 162, 163, 176–177
Amoebozoa, 806
amylase test, 361, 363
Anabaena, 169
anaerobes, facultative and aerotolerant, 91, 359, 609, 621, 628
anaerobic jar, 99–100
anaerobic respiration and fermentation, 91, 287, 295, 333, 401, 627
Ancylostoma duodenale, 820
Animalia kingdom, 783
annelid worms, 178
Anopheles mosquito, 805
anthrax, 216
antibiotics, 417, 529
  disk dispenser, 530
  selective media, 685, 705
  targets and resistance mechanisms, 531
  zone diameter interpretive chart, 531
antibiotic-resistance gene (*bla*), 706, 707
antibodies
  in double gel immunodiffusion, 757–758
  ELISA to detect, 771
  homologous, 753, 757
  structure, light and heavy chains, 751
antigen-antibody interaction, 746, 747, 752, 753, 761
antigens
  A, B, and O in blood typing, 765–766
  ELISA to detect, 771
  purified (PA), 771
  soluble, 753, 757
antimicrobials and antimicrobial susceptibility testing, 417
antimicrobial susceptibility test: disk diffusion method, 529–530
antiseptics, 135
antiserum, 757
api 20 E identification system, 441–442, 445–446
apicomplexans, 803, 805
aquatic habitats, 621, 627. *See also* microorganisms
arabinose operon and digestion, 705, 706, 707
Archaea, 161, 162, 613
Archaeplastida supergroup, 162, 163, 171–172, 782, 801
arginine/arginine decarboxylation, 349, 350–351
arthropods, 178
Ascaris lumbricoides, 818–819
Ascomycota division (Ascomycetes class), 784, 785, 787
  Aspergillus, 788–789
  Candida albicans, 788
  Penicillium, 789
  Saccharomyces cerevisiae, 787
aseptic inoculation, 30
aseptic transfers (slants, broths, plates), 27, 29, 30
  agar slant culture to sterile agar slant, 34, 35, 36
  broth culture to sterile broth, 31, 33–34
  BSL-2 organisms, 31
  Bunsen burner, 30
  fishtail inoculation or streak, 36
  inoculating loops (and needles), 30
  labeling, 29
  nutrient broth pure culture to sterile nutrient broth tube, 32
  "picking" a colony, 37
  plate culture to sterile broth, 36–37
  safety practices, 29–30
  S. epidermidis, 37
aspergillosis, 788–789
Aspergillus, 784–789
  flavus, 789
  fumigatus, 788
  niger, 788, 789
  soyae, 789
  oryzae, 789
  slide culture, 795
assimilatory sulfate reduction, 627
Association of Official Analytical Chemists (AOAC), 121, 135
Astramoeba, 177
ATP/ATPase (adenosine triphosphate) and ATP synthesis, 287, 289, 327, 340, 417, 827
  and sulfur bacteria, 613, 615, 627, 633
autoclaving/autoclave, indicators and tape, 23, 91, 123–124
autoinducer, 633
autotrophs, 83–84, 165, 582, 599
auxochrome, 185
auxotrophs, 733, 782
axopodia, 176
Azotobacter, 589, 591

Bacillaria, 174
bacilli/*Bacillus*, 181, 216, 361
  anthracis, 211, 216, 379
  antibiotic, 641
  cereus, 191, 379
  globigii, 693
  Gram-negative and Gram-positive, 196, 412
  licheniformis, 417
  megaterium, 184, 191, 216

on sheep blood agar, 66
spore-producing, 123, 216
subtilis, 129
surface area, 182
bacitracin susceptibility test, 417–418
bacteria, 169, 782
aerobic, 583, 599
anaerobic, 583, 627
bioluminescent, 633
blood agar to differentiate, 424
citrate-positive, 339
coliform and noncoliform, 235, 647, 648
enteric, 286, 383
environmental factors, 103
fermentation pathways, 340
fungi, 641
gradient, 621
halophiles, 113
heterotrophic, 287
lactose fermenter, 317
marine, 633
mesophile growth range, 103
microaerophilic, 583
nitrogen-fixing, 589, 591–593
oral, 286, 519
osmotic pressure effect, 113
osmotolerant, 113
pH tolerance, 109
in saline habitats, 113
sulfur, 583, 613, 615–617
swimming, 221, 222, 225
temperature effect, 103–104
thermal classifications 103
urease-positive, 383
Bacteria domain, 161, 162, 169, 613
bacteria, sulfur-reducing, exercise, 627–628
bacterial
adhesions, 519
antigens, ELISA to detect, 771
counting techniques, 467
cultural flowchart, 229, 233
cytoplasm, 497
DNA extraction, 689
emulsion, 185, 186
endospores, 124
flagella, 225
incinerator, 31
lawn, 529
motility test, 437
pathogens, 647
smear, 186, 198
staining, 141, 181, 198
transformation, pGLO™ system, 705–707
yogurt species, 676
bacterial cell
DNA extraction, 687
lyses, 497
morphologies and arrangements, 181
walls, 196
bacterial transformation: pGLO™, 705–709
bacterial unknowns project, 457–461
bactericidal and bacteriostatic drugs, 529
bacteriochlorophyll, 613, 615
bacteriophages, 497, 759
Bacteroides/bacteroids, 361, 591, 592
Balantidium coli, 165, 805
Basidiomycota (Basidiomycetes), 784, 785
basic stains, 185
basophils, 745, 746
Beggiatoa, 583, 621, 622
Bevibacillus, 216
BGLB (brilliant green lactose bile) broth, 655, 657, 659
bile esculin agar (BEA), 237, 249–250
biochemical
characteristics, 285
pathways, 827
tests, 285, 286
biofilms, 519, 537–538
biogeochemical cycles/sulfur transformations, 589, 614
biological indicator, 123, 124
bioluminescence, 581, 633–634
in jellyfish, 705
Bio-Rad Laboratories/pGLO™, 705–709
biosafety (BSL) levels, 1, 2, 3. *See also* safety
BSL-1 and BSL-2 safety rules, 3
BSL-2, and precautions, 5–6, 457, 537
aerosol, 31, 846
Alcaligenes faecalis, 110
coagulase precautions, 433
endospore staining, 217
heat-fixing, 187
K. pneumoniae, 211, 385
Moraxella catarrhalis, 85
Mycobacterium smegmatis and *Proteus mirabilis* precautions, 64, 76, 80
Pseudomonas, 225
safety cabinet, 3
transferring microorganisms, 846
Ziehl-Neelsen and Kinyoun method, 205, 206, 207
biosynthesis, 617
biotechnology, 685, 693
blastospores, 784, 788
blocking agent, 771
blood agar/blood agar plate, 423–424, 429, 479
blood typing, 752, 765–767
blood lymphocytes. *See* lymphocytes
bone marrow stem cells, 745, 746
Bordetella, 295
bradyzoites, 806
brain-heart infusion agar (BHIA), 65, 237–238
bright-field wet mount, examples, 183, 621
bromcresol green, 519
bromcresol purple dye, 286, 349
bromothymol blue dye, 273, 286, 295, 340, 345
Brønsted/Lowry theory of acids and bases, 286
broth(s), 30, 33. *See also* nutrient broth
BGLB (brilliant green lactose bile) broth, 655, 657, 659
E-coli (EC), 655, 657
fermentation, 124
fluid thioglycollate, 95–96
growth pattern—flocculence and pigmentation, 79
LTB (lauryl tryptose broth), 655, 657
malonate, 339–340, 345
MR-VP (methyl red and Voges-Proskauer), 303, 311
nitrate, 333–334
nitrate reduction, 610
nitrogen-free, 592, 593
phenol red (PR), 303, 384
urea, 384
broth culture transfer
using digital pipette/pipettor, 843–844
using glass pipette, 839–840
broth tube inoculation with pipette, 841, 844
Brownian motion, 221
BSL. *See* biosafety
budding, 163
Bunsen burner, 29, 30
butyrous colony of soil isolate, 69

Calvin cycle, 615, 617
CAMP test, 429–430
cancer/carcinogens, 685
Candida albicans, 788–789
candidiasis infections, 788
capnophiles, 91
capsule and capsule stain, 211–212
carbohydrate. *See also* lactose fermentation and tests, 303, 401, 519
in milk, 667
oxidation-fermentation test, 295–296
carbolfuchsin, 203, 216
carboxylic acids, 286
casease/casease test, 375–376
casein/casein hydrolysis, 237, 243, 375, 411, 412, 667
catabolic systems, 287
catalase tests, slide and tube, 321–323
cell(s)
acid-fast, 203
arrangements, 181, 184
bacterial, 497, 523
B- and T-, 746
centric and pennate, 173
density, 467
dikaryotic, 784
DNA extraction, 685, 687–590
eukaryotic, 161, 166
Gram-negative and Gram-positive, 195–196, 517
immune, 751
morphologies, 181
natural killer (NK), 746, 747
phagocytic, 751
plasma, 746
surface-to-volume ratio, 182
vegetative, 215
Centers for Disease Control and Prevention (CDC), 121, 517, 541, 665
Cephalosporium, 641
Ceratium, 176
cestodes/cestode parasites, 813, 815. *See also* worms
CFU. *See* colony forming units
charophytes, 163, 171–172
chemical germicides, 138. *See also* germicides
chemical hazard label, 3
chemolithotroph/chemolithotrophic autotrophy, 582, 583, 599, 613, 614
chemolithotrophic sulfur-oxidizing bacteria/energy reaction, 621–623
chemotrophs and chemoheterotrophs, 83, 582
chitin, polysaccharide, 163, 783
Chlamydomonas, 164, 171, 782
chlamydospores, 784
Chlorobaculum, 615
Chlorobium, 615, 628
chlorophytes, 163, 164, 171–172, 173
chloroplasts, 171, 172, 173, 174, 782
CHOPKNSCaFe, 83
Chrococcus, 171
Chromatium, 615
Chromobacterium violaceum, 70, 75
chromogen, 185
chromogenic reducing agent, 327
chromophores, 185
chromoplasts, 173
ciliates/cilia, 165, 175–176, 177
Stentor, 165
citrate medium/citrate lyase/citrate permease, 339
citrate utilization test, 339–340
citric acid (Krebs) cycle, 287, 327, 339, 345
reverse, 615, 616
Citrobacter koseri on sheep blood agar, 67
Cladophora, 172
Clinical Laboratory Standards Institute (CLSI), 417, 529–530
Clonorchis sinensis (Chinese liver fluke), 813
closed system microbial growth, 487
Closterium, 172
Clostridium, 361
acetobutylicum (AGCR), 411
botulinum, 216
difficile, 216

litmus milk to differentiate, 412
perfingens, 216, 379
spore-producing, 216
sporogenes, 66, 411
tetani, 216, 379
clover root nodules, 592
clumping factor, 433
CNS infection, 806
coagulase-reacting factor (CRF), 433
coagulase tube and slide tests, 433–434
cocci
division patterns, 181, 183
surface area, 182
coccobacilli, 181
Cocconeis, 174
coenzymes, NAD^+, NADH, FAD, and $FADH_2$, 287, 327, 328, 340, 345
NADPH, 615
coliforms, 235, 236, 259, 267, 273
multiple tube fermentation method, 655–656
in raw milk, 667
water sample contamination, 647, 648
colistin, 243
colony/colony morphology, 57, 63–64
butyrous colony of soil isolate, 69
effect of age, 66, 70
Enterococcus faecium on nutrient agar, 64
incubation temperature influence on pigment production, 70
mucoid, of *Pseudomonas aeruginosa*, 69
nutrient availability influence on pigment production, 70
soil culture diversity, 71
throat cultures on sheep blood, 71
umbonate, on tryptic soy agar, 68
colony counter, 63, 472
colony-forming unit (CFU), 41, 467, 470–471, 479
density of yogurt unknown, 675
Columbia CNA agar, 237, 243
combination differential media, 393
commensals, 57
competence and sporulation factor (CSF), 215
competitive inhibition, 345
complex/undefined media, 21
compound light microscope. *See* light microscope
candida, 784
Congo red stain, 211
conjugate pair/conjugate base, 286
conjugation, 164
contrast, 181
corynebacterium, **184**
Corynebacterium/*Corynebacterium xerosis*, 68, 181, 183, 198, 361
Cosmarium, 172
countable plates, 470, 497, 500, 641, 648
counterstaining, 195
counting techniques, 467. *See also* soil microbial count
crateriform liquefaction, 379
crustaceans, 178
Cryptosporidium, 204
crystal violet stain and crystal violet-iodine complex, 195, 196, 197
culture
defined, 29
mixed and pure, **41**, 235, 285
tubes, 30
curds, 411, 412
Cyclotella, 173
Cymbella, 174
cynobacteria, 163, 169, 592, 613, 615, 782
cysteine/cysteine hydrolysis, 393, 401
cytochrome and cytochrome c oxidase, 327, 328, 329, 667
cytological stains, 141
cytoplasm/cytoplastic membrane, 171, 176, 182, 185, 237, 327, 497, 784
cytostome, 165

Daphnia, 178
data presentation, (graphs and tables), 9–11
deamination and deamination test, 286, 295, 349, 357, 407, 605
decarboxylation/decarboxylases, 285, 349–351
decarboxylation reactions and tests, 349–351, 407
decimal reduction value (D value), 507–508, 855–856
decolorization, over- and under-, 195, 196–197, 200, 203
decontamination, 121
Deinococcus radiodurans on tryptic soy agar, 68
denitrification, 333, 609–610
dental decay/plaque, 517, 519. *See also* Snyder test
deoxyribonuclease, 367
desmids, 172
desoxycholate, 259
Desulfuromonas acetoxidans, 628
diacetyl, 671
diatoms, centric and pennate 165, 173–175
differential
media, 235, 287, 393
stains, 195, 198, 203, 211, 216
tests, 285, 423
differential blood cell count, 743, 745–747
dinoflagellates, 176
diplobacilli, 181
diplococcus, 183, 184, 197
diplomonads, 163
Diptylidium caninum, 815
direct count, microbial, 483–484
diseases. *See also* pathogens
hydatid, 816
infectious, 541, 543
disinfection/disinfectants, 121, 135
disk diffusion method, 417, 529
dissimilatory sulfate reduction, 627
DMABA, 394
DMSO (dimethyl sulfoxide), 733–734
DNA
binding protein *ara*C, 705, 707
cell extraction, 687–690
copying *in vitro*, 685
gyrase, 417
hydrolysis, 367–368
mutations, 685
photolyase, 727
repair, light and excision, 727
synthesis, 237
viral, 497
DNA polymerase chain reaction 713–716
DNase test and DNase test agar, 361, 367–368
domains, eukaryotes and prokaryotes, 162
double gel immunodiffusion, 752, 757–758
Durham tube examples, 286, 333–334. 336, 610, 657

Echinococcus granulosus, 816
E. coli (*Echerichia coli*), 237, 311, 327, 328, 395, 497, 551, 647, 655
broth, 655, 657
competence through GFP, 705–706
DNA, 685, 687–690
excision (dark) repair, 727
identification, of bacteriophage infection, 685
illnesses, 665
phage typing, 739–740
photoreactivation/photolyase, 727
*Eco*RI, 693
Ectothiorhodospira, 615
electromagnetic energy/spectrum, 129, **130**
electron(s)
acceptor, nitrate as, 609
as energy source, 582
lactate donor, 628
microscopy, 141, 195
tower, 289–290
electron transport chain (ETC), 287, 290, 321, 615
Complexes I, II, III, and IV, 327
methylene blue, 667
electrophoresis/gel electrophoresis, 857–858
ELISA test, 743, 752, 771–776, 802, 821
Elvers and Lappin-Scott, biofilm definition, 537
Embden-Meyerhof-Parnas pathway, 827–830
amebic dysentery (amebiasis), 806
endospores/elliptical endospores, 215, 216
endospore stain, 215, 216, 218
Schaeffer-Fulton, 217
steaming the slide, 218
endosymbiosis, 165, 782
energy metabolism, 285, 827
energy of formation, 289
entamoebas, 163, 806
Entamoeba coli, 807
Entamoeba histolyca, 806–807
Enterobacteriaceae, 235, 259, 261, 367, 395. See also *E.coli, Morganella, Proteus, Providencia, Salmonella, Shigella*
aerobic respiration, 328
aerogenes, 647
api 20 E system, 441–442
citrate utilization test, 339–340
decarboxylation and deamination tests, 349, 350
enzymes, 349
on eosin methylene blue agar, 267
gelatinase test, 379
on hektoen enteric agar, 273
IMViC test battery, 395
lactose fermentation, 647
litmus milk to differentiate, 412
on MacConkey agar, 259, 261
motility, 395, 437
on MR-VP broth, 312
nitrate reduction test, 333
O-F test, 295
ONPG test, 318
oxidase test, 328
sulfur-reducing, 628
TSIA and KIA to differentiate, 402
on xylose lysine desoxycholate agar, 279–280
Enterobius vermicularis, 819
Enterococcus, 361, 389
blood agar to differentiate, 424
faecalis (ACR), 411
faecium on nutrient agar, 64
methylene blue reductase test, 667
EnteroPluri-*Test* (Enterotube® II), 449–451
Entner-Doudoroff pathway, 303, 827, 829, 830
entropy, law of, 289
environmental/environment
aquatic, 592, 621, 627

factors affecting microorganisms, 55, 85, 91, 103
microbiology/microbes, 581
trophic groups, 582
Environmental Protection Agency (EPA), 121, 135
enzymes, 341
aminopeptidases, 389
in arabinose digestion, 705
β-galactosidase, 317, 411–412
casease, 375
citrate lyase and citrate-permease, 339
coagulase, free and bound, 433
Complexes I, II, III, and IV, 327
conjugated, 771
cysteine desulfurase, 393
DNase, 367, 417
Enterobacteriaceae, 349
exoenzyme, 361
extracellular and intracellular, 361
horseradish peroxidase (HRP), 771
hydrolytic, 285, 361
luciferase, 633
lysozyme, 517, 523
nitrate reductase, 333
nitrogenase, 589
proteolytic, 412
restriction, 693–694
thiosulfate reductase, 393
tryptophanase, 395
yogurt, 671
enzyme-coenzyme-substrate complex, 287
eosin methylene blue (EMB) agar, 259, 267–268
epitopes, 752
Erwinia amylovora on nutrient agar, 67
Escherichia coli. See *E.coli*
esculin/esculin hydrolysis, 248
eosinophils, 745
erythrocytes, 745
eosinophils, 745, 746
ETC (electron transport chain), 287, 295, 327
ethidium bromide (EtBr), 715
euglenozoans/euglenids, 163, 164, 176
"eyespot," 164, 176
flagella, 164, 176
Eukarya domain, 162, 782, 901
eukaryotes/eukaryotic microbes, 103, 161, 162, 166, 327, 613
medically important, 781
eukaryotic supergroups
Archaeplastida, 163–164, 169, 801
Excavata, 162, 163, 164, 169, 801
SAR, 164–165, 169, 801
Unikonta, 163, 169, 176–177, 782, 783, 801
Excavata supergroup, 162, 163, 176–177, 782, 783
diplomonads, parabaslids, and euglenozoans, 801
Giardia lamblia, 801–802
pathogens, 801–803
Trichomonas vaginalis, 802
Trypanosoma brucei, 802–803
excystation, 807
exoenzymes, 163, 361, 783
experimental design/experimental variable, 8–9
cause and effect relationship, 8
limitations (false positives and false negatives), 8–9
sensitivity and specificity equations, 9
extracellular polysaccharide, 537

Fabaceae, 591
FDA, GRAS (generally regarded as safe), 789
FEA (final electron acceptor), 287, 297, 303
fecal contamination, 235, 259, 647
fermentation, 286, 287, 833
alcohols produced by, 286
broth, 124
carbohydrate, 285, 303, 401, 519
citrate, 339–340
end products, 287
eosin methylene blue agar, 267–268
gas-producing, 286
glucose, 303, 349, 401
Krebs cycle, 830
lactose, 235, 236, 259, 261, 317, 401, 647, 648
MacConkey agar, 259
mannitol salt agar, 253
of milk sugar lactose (yogurt making), 671
mixed-acid, 311
multiple tube method for determining water quality, 655–658
pathways, 833, 834
Penicillium, for cheese, 789
of sugars, 517
tests, 303
xylose lysine desoxycholate agar, 279
fermenters
anaerobic bacterial, 627
large-scale manufacturing, 467
ferric ammonium citrate, 273, 279, 407
ferrous sulfate, 401
flagellates/flagella
amphitrichous, 225
dinoflagelate, 176
Euglena, 164
Excavata. 782, 801
lophotrichous, 225
mixotropic, 176
peritrichous, 225
polar, 225
stain, 225–226
Unikonta, 783
flaming loop, 33
flatworms, 177, 813
flavin mononucleotide ($FMNH_2$), 633
flavoproteins, 327
Fleming, Alexander, 523, 789
fluid thioglycollate broth (FTB), 95–96
flukes, 813, 814
Food and Drug Administration (FDA), 121, 135, 665
food microbiology, 665
Fragillaria, 174
free energy, 289
frustule, 173
fucoxanthin, 165, 173
fucsin, 648
fungal slide culture/conidiophores, in Penicillum and Aspergilus, 795–797
fungi/Fungus
Ascomycota, 785
Basidiomycota, 785
Candida albicans, 788
dimorphic, 783
life cycles/reproduction, 784
macrofungi, 785
medically and economically important, 781, 782
pathogens, 641, 788–789
soil microbial count, 641
unicellular yeasts and filamentous molds, 163
in Unikonta supergroup, 782
Fusobacterium, 361

galactose, 676
gametangia, 784, 786
Gardnerella vaginalis, 361
GasPak® Anaerobic System by BBL, 99–100
gastrotrich, 177
gelatinase/gelatin hydrolysis test, 361, 379–380
genes, structural (operon), 705
genetic engineering, 685, 693, 705
Geobacillus, 216
stearothermophilus, 123
germicides, 121, 135–136, 138
Giardia lamblia (intestinalis), 801–802
giardiasis, 801
Giemsa stain, 745, 746
glass pipettes, transfer from broth culture, 839
glucose
for ATP production, 327
fermentation, 303. 349, 401
oxidation, 827–830
in Snyder test, 519
in yogurt making, 671
glycocalyx, 537
glycoproteins, 751
glycolysis, 287, 303, 312, 327, 827–830
glycolytic pathway, 671
Gomphonema, 175
Gram-negative and Gram-positive cells and organisms, exercises. *See also* Gram stain
api 20 E identification, 441
Azobacter, 591
bacteria, 333, 448
bacterial unknowns, 457–458
cocci, 433, 517, 519
Columbia CNA, 243
EnteroPluri-*Test*, 448
hemolysins, 423
lysozyme assay, 523
MacConkey agar, 259–261, 442
medical biology, 517, 519
membrane filter, 648
nitrate reduction test, 333
PEA, 237–238
selective media, 235
Gram-positive rods, identification, 517, 573–577
Gram stain, 181, 182, 183–184, 185, 195–197, 198
bacteria, 591–592
E. coli, 647
gumline, 519
procedure, 200
yogurt, 677
granulocytes, 745
green algae, 164, 169, 171
green fluorescent protein (GFP), 705–706, 707
green sulfur bacteria, 615, 616
Griffith, Fred, 705
growth media, 21
in autoclave, 91
growth patterns in broth, 79, 95
guanidine, 311
Gymnamoebas, 163

Hafnia, 395
halophiles, 113
hand-cleaning agents, 15, 17, 18
hanging drop preparation, 221–222
hazardous waste disposal, 124
health-care associated infections (HAIs), 537
heat-fixing, 186, 187, 191, 211
Helicobacter pylori, 380, 384
helminths, parasitic, 781, 813. *See also* worms
hemagglutination, 765
hektoen enteric (HE) agar, 259, 273–274
heliozoans/*helia*, 176
hemagglutination, 752
hemoglobin, 423, 592
hemolysis
aerobic versus anaerobic, 424
α-hemolysis, β-hemolysis, γ-hemolysis, 423, 424
HEPA filter, 3
heterocysts, 169, 170, 592
heterotrophs, 83, 84, 163, 582, 627, 782
absorptive, 783

hookworms
Ancylostoma duodenale, 820
Necator americanus, 820
Hugh and Leifson's O-F medium, 295
Hydrodictyon, 172
hydrogen peroxide/hydrogen peroxide substrate, 321, 771
hydrogen sulfide, 393, 401, 407, 621
hydrolytic reactions/enzymes, 285, 361
Hymenolepis nana, 817
hyphae
Penicillium, 789
pseudohyphae, 788
septate and nonseptate, 783, 784

immune system/immunity, 743, 751
immunodiffusion, double gel, 757–758
immunoglobin, 746, 771
IMViC test battery, 312, 340, 395
indicator dyes, 286
milk quality, 667
indicator reactions (antibody/antigen), 753, 771
indicators
E. coli, 647
selective media, 236. *See also* pH
indole production (IND), 393–395, 444
inhibitors, selective media, 236
inoculating instruments and methods, 27, 29–30, 33, 75, 437, 621, 797, 836. *See also* aseptic transfers
invertebrate animals, 177–178
iodine, 361
iron-sulfur protons, 327
isolation method, streak plate, 41
Isopora, 204

jellyfish, 705, 706

karyogamy, 784
keratin, 215
kinetoplastids, 163
Kinyoun method, 203, 204
Kirby-Bauer method (Alfred Bauer and William Kirby), 529–530
Klebsiella
antibiotics, 243
citrate utilization, 340
IMViC battery, 395
pneumonia, 67, 647
Kligler iron agar (KIA), 401–402
Kocuria rosea
on brain-heart infusion agar, 65
on slant, 75
Köhler illumination/August Köhler, 147–148
Kovac's reagent, 394
Krebs cycle, 287, 830, 832, 833

Lacobacillus, 519, 676, 677
delbrueckii, 671
lactis, 182, 411
plantarum, 75
lactose, 671
fermenters and non-fermenters, 235, 236, 259–260, 267, 273, 286, 303, 317, 401, 647, 648
hydrolysis, 317, 412
in milk, 667, 411
leghemoglobin, 591–592
leprosy, 203
leukocytes, 745, 746
light microscope/light microscopy, 143, 145. *See also* Köhler illumination
amoeba micrographs, 143
bright-field, 143
compound, 144, 145
dark-field, 143, 145
fluorescence, 143, 145
instructions for using, 145–146
magnification formula, 143
Mycobacterium kansasii, 143
numerical aperture, 144
objective lens, 144
apochromatic, 144
oil-immersion, 144
ocular lens, 143
phase contrast, 143, 145
refraction, 143
resolution/resolving power, 143–144, 145
virtual image, 143
lipase test, 371–372
lipid hydrolysis, 372–373
litmus milk, 411
Lophotrichnous flagella, 225
LTB (lauryl tryptose broth), 655, 657
luciferase, 633
lymphatic inflammation, 821
lymphocytes, B- and T-, 745, 746, 747, 751
Lyngbya, 169
lysine and lysine decarboxylation, 259, 349–350
lysine iron agar (LIA), 407–408
lysis, cells, 497, 523
lysozyme, and lysozyme assay 517, 523–524
lytic cycle/lysis, 497

MacConkey agar, 259–261, 442, 479
macronucleus, 165
macrophages, 746
magnification, 181
malachite green, 215
malaria, 805
malonate utilization test, 345
mannitol salt agar, 237, 253–254
marine bacteria. *See* bioluminescence
mastax, 177
McFarland turbidity standard, 529–530
media. *See also* differential tests; selective media
combination differential, 393
complex/undefined, 21, 84
culture, 29
defined and chemically defined, 84
differential, 235
labeling, 29
plated, 361
utilization, 339
medical microbiology, 517
meiosis, 163, 164, 784, 785
membrane filter technique/apparatus, 647–651
Merismopedia, 170
mesophiles, 103
metabolic pathways/reactions, 285, 286, 327, 705, 830
methanogens, 627
methicillin-resistant *Staphyloccus aureus* (MRSA), 529
methionine, 627
methylation/methylase enzymes, 693
methylene blue, 715
reductase test, 667
methyl red and Voges-Proskauer (MR-VP) tests, 311–312, 313, 395
microaerophiles, 91
microbial/microbes
fermentation products, 286
population density, 467
soil, 637, 641–642
microbial growth
in broth, 79–80, 95–96
closed system (lag, log, and death phases), 487–488
direct counts, 483
environmental factors, 83, 91
in oral cavity, 519
osmotic pressure effect, 113
pH effect, 109–110
plate counts, 483
on slants, 75–76
temperature effect, 103–104
ultraviolet radiation lethal effect, 129–130
microbial identification
of morphological unknown, 229
susceptibility testing, 417
traditional methods, 517
microbiology
environmental. *See* environmental microbiology
eukaryotic microscopic organisms, 781
food. *See* food microbiology
identification methods, 739
Micrococcaceae, 322
Micrococcus luteus
on brain-heart infusion agar, 65
negative stain, 191
on slant, 75
tetrad arrangement, 184
Micrococcus lysodeikticus, 523
Micrococcus roseus, 197
Microcystis, 170
micrometers, calibration
calibration, 155–156
hemacytometer, 155
ocular, 155–156
stage, 155–156
micronucleus, 165
microorganisms/microbes. *See also* agar deep stabs; agar slants; colony/colony morphology; microbial growth
antimicrobials, 417
Beggiatoa, 583
biosafety levels, 1–3
commensals, 57
control agents and systems, 55
culture, mixed, 57
diversity, 55, 57
environmental factors affecting, 55, 85, 91,103
free-living, 57
growth characteristics, 55
history, 57
pathogenic and nonpathogenic, 57, 531
planktonic, 537
reservoir, 57
salinities, 113
soil, 641–642
sulfur, 613, 621
symbiotic, 57
temperature effect, 103–104
ubiquity, 57–58
microsope, binocular compound, 142
microscopy, 141. *See also* light microscope
phase contrast, 144
milk agar, 361, 375. *See also* litmus milk
milk quality tests, 655
minimum inhibitory concentration (MIC), 417, 529
molds/filamentous molds, 163
bread, 784, 785, 787
slide cultures, 795
molecular taxonomy/molecules, 685, 752
Møller's decarboxylase base medium, 349, 350
monocytes, 745, 746, 747
Moraxella/Moraxella catarrhalis, 85, 295, 329, 367
Morbidity and Mortality Weekly Report (MMWR) report, 541–543
Morganella/Morganella morganii, 357, 383, 395
mordant, 225
morphological unknown, identification, 229
morphologies, cell, 181

motility, 221
in SIM, 393–395
test, 437–438
MPN method for determining water quality, 655–657
MR-VP broth, 303
mud samples. *See* water
Mueller-Hinton agar, 529
Mullis, Kary, 713, 715
multiple test systems, 441
mushroom/macrofungus, 783
mutagenicity, testing for, 735
mutation, 685, 733 (frameshift, back, missense substitution)
mutualism, 57
Mycobacterium, 203, 204
identification, through catalase test, 322
kansasii, 143
phlei, 75, 203
smegmatis, 64, 68, 76, 80, 204
ZN method, 204
mycolic acid, 203

NAD^+ and NADH, 287, 827. *See also* ATP synthesis
nalidixic acid (CNA), 243
National Yogurt Association (NYA), 675, 677
natural killer (NK) cells, 746, 747
Navicula, 175
Necator americanus, 820
negative stains, 191–192
Neisseria, 328, 361, 367
sicca, 198
Nemotoda/nematode worm, 177, 813
Ascaris lumbricoides, 818–819
Enterobius vermicularis, 819
neutrophiles, pH tolerance, 109
neutrophils (segs, PMNs, and bands), 745, 746
nigrosin acidic stain, 191, 211
nitrate reduction test, 333–336, 442, 444, 609–610
nitrification, and nitrate test 599–601
Nitrobacter, 599
nitrogen cycle, 581, 583, 589
nitrogen fixation (N-fixation), 589, 591–593
Nitrosomonas, 599
Nitzschia, 174
Nocardia brasiliensis and *asteroides*, 204
Nostoc, 170, 589
novobiocin susceptibility test, 417–418
nutrient agar, 21, 22–24, 243, 253, 259, 641
nutrient broth, 21, 22, 23–24
nutrient utilization tests
Malonate utilization, 339, 345
Simmons Citrate Medium, 339–340

obligate aerobe, 401
ocular micrometer, calibration, 155–156
hemacytometer, 155
stage, 155–156
Oedogonium, 171
ONPG test, 317–318
β-galactosidase conversion, 317
oocysts, 204
operons, arabinose, 705–706
Opisthokonta, 785
optochin susceptibility test, 417–418
oral cavity. *See* Snyder test
original cell density (OCD), 479, 483–484
ornithine/ornithine decarboxylation, 349
Oscillatoria, 170
osmosis/osmotic pressure/osmotic homeostasis, 113, 237
oxaloacetate, 339
oxidase test, 327–330, 441
oxidation, 289, 614
pyruvate, 830
oxidation-fermentation (O-F) test/medium, 295–296
oxidation-reduction (redox) reactions, 287, 289, 442
oxidative phosphorylation, 287, 321
oxidized iron, 236
oxygen, aerobic and anaerobic, 91

Paenibacillus, 216
palindromes, 693
parabasalids, 163
paragonimiasis, 813
Paragonimus westermani, 813–814
Paramecium, 175
bursaria, 165
parasites. *See also* worms
animal (apicomplexans), 803
cestode, 815–818
coccidian, 204
entamoebas, 163
fungi, 783
hookworms, 820–822
nematode, 818–819
parasitic helminthes (worms), 781, 813
trematode, 813–814
pathogens, 57. *See also* worms
alveolate, 803–806
bacterial, 647
Candida albicans, 788
disk diffusion test, 521
Excavata, 801 –803
fungi, 641, 788–789
germicidal systems, 121
Helicobacter pylori, 380
in milk, 667
Mycobacterium leprae and *tuberculosis*, 203
Nocardia, 204
opportunistic, 788–789
selective media to determine, 235, 259
Staphylococcus aureus, 433
Toxoplasma, 806
Unikont, 806
waterborne, 647
PCR, 713, 718–719, 806. *See also* polymerase chain reaction
Pediastrum, 172
pellicle, 79, 519
penicillin, 789
Penicillium, 641, 788
commercial cheeses, 789
conidiophores, 789
marneffei, 789
notatum, 789
slide culture, 795
pentose-phosphate reactants, 827, 828, 829, 831
peptidoglycan, 196, 197, 417, 523
Performance Standards for Antimicrobial Testing (CLSI), 417
petri dish and mask, examples, 29, 728, 796
Petroff-Hausser counting chamber, 483
pGLO™ system, 705–707
transfer pipette, 710
pH
in biochemical tests, 286
in citrate utilization test, 340
effect on microbial growth/scale, 109–110
fucsin to indicater, 648
homeostasis, 237
in lysine iron agar, 407
in MR-VP broth, 311
Søren Peter Luritz Sørensen, 109
triple sugar iron agar, 401
phage typing, 739–740
phagocytic polymorphonuclear granulocytes (PMNs), 523
phagocytosis, 745
phase contrast images, sulfur oxidizer and reducer, 623, 628
phenol red, 253, 286, 383, 384, 401, 401
phenol red broth , 303, 384
phenylalanine, 349
phenylalanine deaminase test, 357–358
phenylethyl alcohol agar (PEA), 237–238
pH indicators, 124, 259, 286, 349, 401, 412, 519. *See also* fermentation
photoautotrophs and photoheterotrophs, 582, 583, 613, 614
Photobacterium, 633
photolysis, 592
photoreceptors, 177
photosynthesis by cyanobacteria, 592
photosystems, 615
phototrophs/phototrophic organisms, 83, 583
sulfur oxidizing anoxygenic, 615
pipette/pipettor
calibration, 839
digital, 846, 843–844, 852, 853
disposing, 841
filling, 840–841
glass, 839
inoculation of agar plates, 842
inoculation of broth tubes, 841
mechanical, 839
Mohr, 840
serological, 840
planarian, 177
plaque assay technique/plaque forming unit (PFU), 497–500
alternative procedure, 853–854
plasma cell, 746
plasmid, pGLO™, 706, 707
Plasmodium spp. (*vivax, falciparum, malariae, ovale*), 803–805
plasmogamy, 784
plasmolysis, 113
plates. *See* agar plates
Platyhelminthes (flatworms), 813
pleomorphism, 181, 183
pneumonia, *Ascaris*, 818
polymerase chain reaction (PCR), 713–716
polysaccharides, 361, 751
population density, 467
potassium phosphate, 383
pour-plate technique, 497
precipitation reactions/precipitation ring test, 743, 752, 753–754
in vivo and in vitro, 757
prokaryotes, 113, 161, 162, 827
Prosthecochloris, 615
proteins, 497, 751
progametangia, *Rhizopus*, 786–787
Proteus
antibiotics, 243
gelatin hydrolysis test, 379
mirabilis, and precautions, 64, 76, 80, 290
PEA with, 237
phenylalanine deaminase test, 357
SIM media, 395
swarming behavior, 69, 225
urea hydrolysis, 384
vulgaris flagella, 225
proton, 327
proton motive force, 615
prototrophs, 733
protozoans, as waterborne pathogens, 647
Providencia
on nutrient agar, 67
SIM medium, 395
on urea agar, 383
on XLD agar, 259, 279

pseudomonadaceae, oxidase test to differentiate, 329
Pseudomonas/Pseudomonas aeruginosa
antibiotics, 243
diffusible pigment, 70
disinfectants against, 135
flagella, 225
litmus milk to differentiate, 411
mucoid colonies, 69
O-F test, 295
on slant, 75
TSIA and KIA to differentiate, 402
Pseudomonas denitrificans, 609
pseudopods/pseudopodia, 163, 176, 177, 782, 806
"pseudovacuoles," 170
psychrophiles and psychrotrophs, 103
purple sulfur bacteria, 615, 617
pyrenoids, 171, 172, 173
pyrimidine dimers, 727
PYR test, 361, 389–490
pyruvates, 287, 295, 303, 327, 339, 340, 627, 671, 827, 830

quorum sensing, 633

raphe, 173, 174
red blood cells (RBCs), 423, 745, 752, 765
redox pairs/reactions, 289–290, 615
reduction potential, 289–290, 327
reproduction
asexual, 172, 784
sexual, 173, 784
resolution, 181
respiration, aerobic and anaerobic, 287
restriction enzymes/endonucleases, 693–694, 696
reverse citric acid cycle, 615, 616
reversion in slants, 401
Rh factor, 766
rhizarians, 176, 782
Rhizobium
leguminosarum on brain-heart infusion agar, 68
nitrogen-fixing bacteria, 589, 591, 592
Rhizopus, 785–787
life cycle, 786
microsporus, 786
oryzae, 786
progametangia, 786
rhizoids, 785
sporangiophores, 786
stolonifer, 785, 787
zygosporangium, 787
Rhodococcus rhodochrous on brain-heart infusion agar, 65
Rhodospirillum rubrum, 191
Rivularia, 170
RNA polymerase, 705
rotifers, 177
roundworms, 177, 818
ryu stain, 226

Saccharomyces cerevisiae, 163, 783, 787
safety, laboratory, 3–5, 29–30. *See also* biosafety
in aseptic transfers, 29
autoclave and autoclave bag, 6, 7
chemical hazard label, 3
contamination, reducing and disposing, 5–7
petri dishes, disposal, 7
protective clothing and tube holder, 4
sharps container, 4
sterilizers, temperature, 123
student conduct, 3
taping plates, 6
safranin, 195
Salmonella
agglutination test, 761–762
Ames test, 733
enterica serovar Cholerasuis, 135
on HE agar, 259, 273
on LIA, 407
on methyl red, 311
serotyping, 752
SIM medium, 395
testing for mutagenicity, 733
typhi (typhoid fever), 647
on xylose lysine desoxycholate agar, 279
saltern, 114
saprophytes, 57, 163, 216, 641, 783
sarcina, 181, 183
Sarcina aurantiaca on brain-heart infusion agar, 65
SAR supergroup (stramenopiles, alveolates, and rhizarians), 162, 164–165, 173–176, 782, 801
Scenedesmus, 172
Schaeffer-Fulton endospore stain, 215, 217
Schistosoma mansoni, 814–815
sediment, 79
selective media, 235, 236
antibiotic, 685
for isolation of gram-negative rods, 259
for isolation of gram-positive cocci, 237
for soil sample assessment, 581 593
semipermeable membrane, 113
serial dilution/dilution blanks, 467, 469, 497, 676, 852
serological reactions/tests, 743, 752, 771. *See also* antibodies; antigens
Serratia, 367, 379, 395
marcescens, 41, 70, 75, 693
Shigella
dysenteriae (dysentery), 647
on HE agar, 259, 273
on LIA, 407
on methyl red, 311
on SIM media, 395
on xylose lysine desoxycholate agar, 279
SIM medium, 393–394
Simmons citrate medium/slant, 339, 340
simple stains, 181, 185–186, 187
slants. *See also* agar slants; Simmons citrate
growth patterns, 75–76
KIA, 401–402
LIA, 407–408
slide agglutination test, 752, 761–762
smears, bacterial, 185, 187, 197, 198
Snyder test, 519–520
sodium chloride, 236
sodium thiosulfate, 279, 407
soil
fertility, 581, 591–593
microbial count, 641–642
slide culture, 637–638
Sørensen, Søren Peter Lauritz, and pH formula, 109
spectrophotometer, 488, 490, 847–849
spirilla, 181, 182, 183
Spirilum voluntans, 225
spirochetes, 181, 183
Spirogyra, 164, 172, 173
Spirulina, 170
sporangia/sporangiospores, 784, 785, 786
sporulation, 215
spot inoculations, 235
spread plate method of isolation, 49–51
turntable, 50
SRB. *See* sulfate reducing bacteria
stains/staining, 185
acid-fast, 195, 203–204
acidic, 191, 211
basic, 185
capsule, 211–212
confirmatory test, 229
Congo red, 211
cytological, 141
differential, 195, 198, 203, 211
endospore, 215–216
flagella, 225–226
Gram, 181, 182, 183–184, 185, 195–200
Kinyoun (K), 203, 204
negative, 191–192
nigrosin, 211
precipitate, 198
ryu, 226
Schaeffer-Fulton, 215
simple, 181, 185–187
structural, 195
technique, 197, 198
Ziehl-Neelsen (ZN), 203, 205–206
standard plate count, 469–471, 851
staphylococci/*Staphylococcus*, 183
bacitracin test. 418
aureus, 71, 135, 184, 253, 367, 379, 429, 433, 529, 531, 537
biofilm, 538
epidermidis, 65, 75, 204, 379, 423, 537
Gram-positive and Gram-negative, 196, 237
mannitol salt agar, 253–254
PEA isolation, 237
saprophyticus, 418
sterilization, 121
steam, 123–124
starch and starch agar, 361
starch hydrolysis test, 361–362
stramenopiles, 165
stratified squamous epithelium, 519
streak
fishtail, 401, 407
pattern on agar plate, 58
semiquantitative method, 479
urine, 479
streak plate isolation methods, 235
quadrant streak method, 41–42, 45
Serratia marcescens, 41
T-streak method, 42–43, 45
zigzag inoculation of agar plates using cotton swab, 43–44, 45
streak-stab technique, 424
Stentor, 165
streptobacillus, 181, 184
streptococci/*Streptococcus*, 181, 182, 183
agalactiae, 429
bacitracin test, 418
blood agar to differentiate, 424, 479
bovis, 249, 250
catalase-negative, 322
disk diffusion, 417
equinus, 250
gallolyticus, 250
mutans, 519
oral, 519
PEA test, 237
pneumoniae, 211, 417, 418, 423
pyogenes, 423
PYR test, 389
salivarius, 184, 383
in yogurt, 676, 677
streptolysins O and S, 424
Streptomyces, 641
griseus, 68
niveus, 417
Strongyloides stercoralis, 821
structural stains, 195
Student Safety Contract, 13

substrates, 236
substrate-level phosphorylation, 287, 827
succinate dehydrogenase, 345
sucrose, 259, 401
sulfate reducing bacteria (SRB) (*Desulfobacter, Desulfobulbus, Desulfococcus, Desulfonema, Desulfosarcina, Desulfotomaculum, Desulfovibrio*), 627, 628
sulfide, 614, 627
sulfur
  bacteria, 583, 613
  cycle and compounds, 581, 613
  oxidizers, 613
  reduction and fermentation, 393–395, 401, 407, 614, 627
sulfur bacteria, photosynthetic, 615–617
sulfur-oxidizing bacteria, chemolithotrophic, 621
sulfur-reducing bacteria, 627–628
supergroups, eukaryotic, 161, 162
  Archaeplastida, 163–164, 782, 801
  Excavata, 163, 164, 176, 782, 801
  SAR, 164–165, 173–175, 782, 801
  Unikonta, 163, 176, 782, 801
superoxide dismutase, 321
susceptibility tests—bacitracin, novobiocin, and optochin, 417–418
syntrophic communities, 613

Tabellaria, 173
tachzoites, 806
Taenia
  saginata, 817
  solium, 817
tapeworms, 813
  Dipylidium caninum, 815
  Echinococcus granulosus, 816
  Hymenolepis nana, 817
  Taenia spp., 817
T-cell, 746
test tube rack, 4, 30
tetrad, 181, 183
tetrazolium salt (TTC), 437
thermal death time (TDT), 507–508, 855–856
thermophiles, 103
Thermus aquaticus, 715
Thiobacillus (*acidophilic, denitrificans, ferroxidans, novellus, thiooxidans, thioparus*), 621
Thiopedia, 615
Thioplaca, 621
Thiospirillum, 615
thiosulfate/thiosulfate reductase, 393, 401, 407, 627
Thiothrix, 621
tooth biofilm. *See* Snyder test
Toxoplasma gondii, 805–806
toxoplasmosis, 805–806
Trachelocerca, 175
transfer methods
  cotton swab, 835–836
  spot inoculation of agar plate, 837
  stab inoculation of agar tubes, 836
trematodes, 813
  Clonorchis sinensis (Chinese liver fluke), 813
  Paragonimus westermani, 813–814
  Schistosoma mansoni, 814–815
Treponema, 191
Tributyrin agar, 361
Trichomonas vaginalis, 802
trichomes, 169, 170
triple sugar iron agar (TSIA), 401–402
trophozoites, 806
tropic groups, 582, 583
Trypanosoma brucei, 802–803
tryptophan hydrolysis/tryptophan deaminase (TDA), 394, 444
tuberculosis, 203
tubes, microgradient, 621, 622. *See also* inoculating
turgor pressure, 113

Ulothrix, 171
ultraviolet (UV)
  bacterial effect of radiation, 685
  fluorescence, 705
  spectrophotometer, 687
ultraviolet radiation and protection, 129–130
ultraviolet radiation damage and repair, 727–728
umbonate colonies of anaerobic lab contaminant on tryptic soy agar, 68
undecaprenyl phosphate, 417
Unikonta supergroup, 162, 163, 176–177, 782, 783, 801
urea hydrolysis/urease test, 361, 383–384
urine culture and streaks/urinary tract infection, 479
use-dilution test, 135
U.S. Food and Drug Administration, 529
utilization media, 339

vacuoles, 172
vector, 705
vegetative cells, 215
viable (plate) count, 469, 665
  alternative procedure, 851–855
  in food industry, 675, 677
vibrio/*Vibrio*, 181, 183, 633
  cholerae (cholera), 647
  natrigens, 488
viral
  antibodies, ELISA to screen, 771
  DNA and proteins, 497
  population, measuring, 467
Virgilbacillus, 216
Viruses, 739
Voges-Proskauer (MR-VP) test, 311–312, 313, 395, 444
volumetric loop, 479
Volvox, 164, 171, 782
vortex mixer, 33
Vorticella, 175

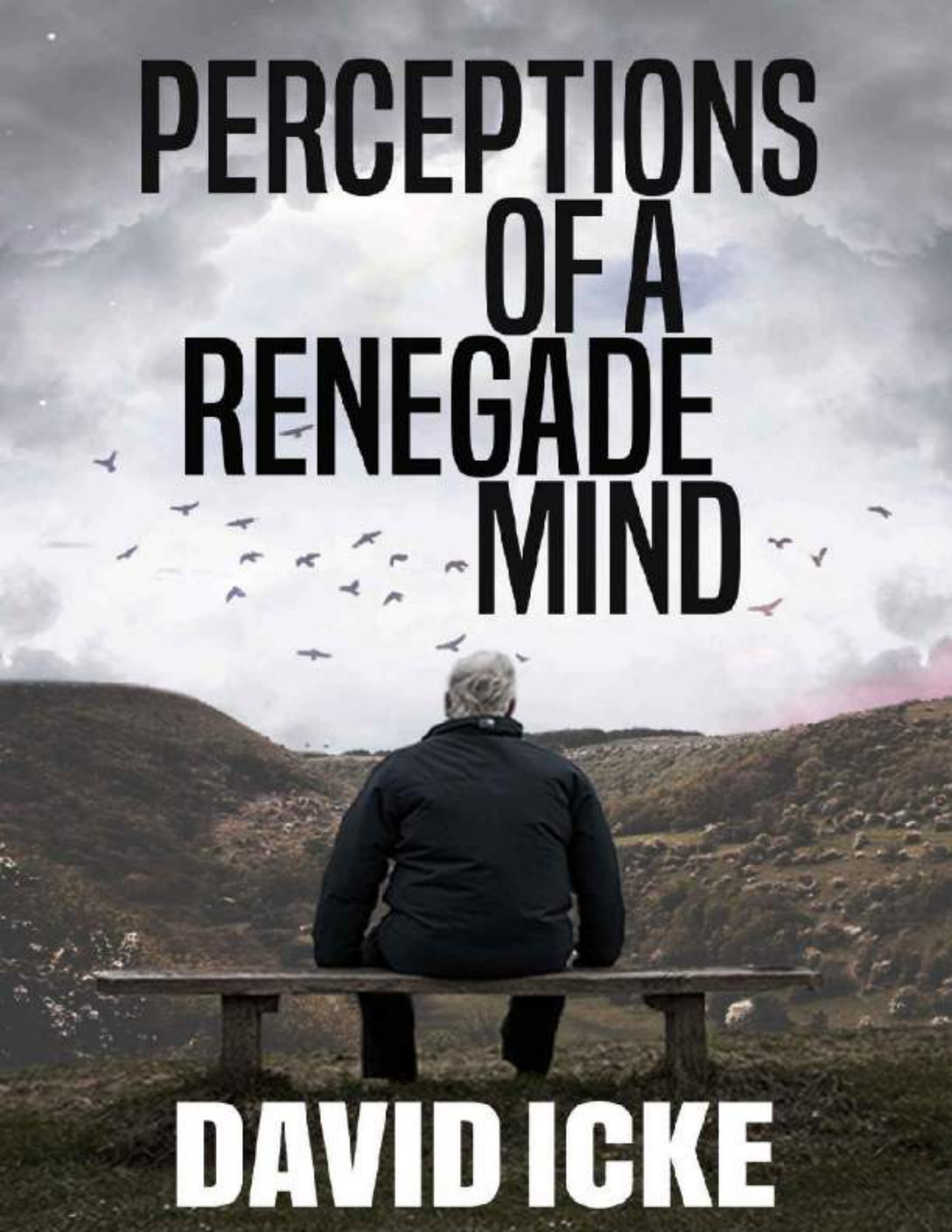
water
  diffusion, 113
  and mud samples, 622
  pollution, fecal, 647
  potability, 581, 648
  quality testing, 647, 655
wavelength, three groups, 129
wet mount, examples, 162, 221–222, 591, 621, 789
white blood cells (WBCs), 745, 746
Winogradsky column (Sergei Winogradsky), 581, 583–585, 615
World Health Organization (WHO), 121
worms
  annelid, 178
  filarial, 821
  flatworms, 813
  hookworms, 820–821
  nematodes, 177, 818–819
  parasitic, 781, 782, 813, 821
  pinworms, 819
  tapeworms, 815–818
  threadworms, 820–821
  trematode, 813–814
Wright's stain, 745, 746, 747
Wuchereria bancrofti, 821

xylose lysine desoxycholate (XLD) agar, 259, 279–280

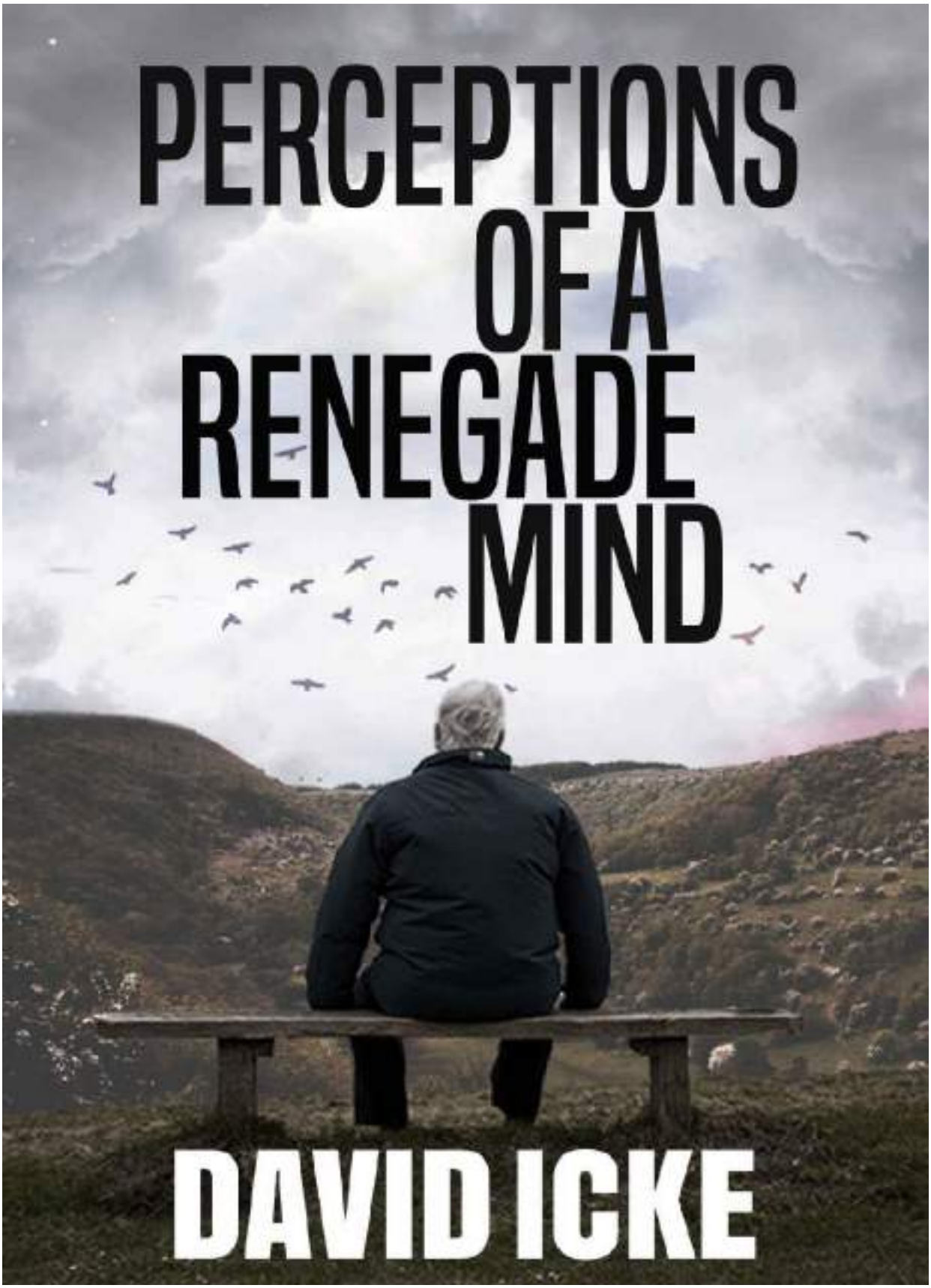
yeasts
  ascomycete, 784
  brewer's, 163, 783
  extract, 236, 243, 407
  unicellular, 163
yogurt, 665
  bacterial species, 676, 677
  CFU density of unknown, 675–676
  making, 671–672

Ziehl-Neelsen (ZN) staining method, 203, 205–206
Zone Diameter Interpretive Charts, 417, 531
zone of equivalence, 753
zone of inhibition, 417, 529, 734
zygomycosis, 786
Zygomycota, 164, 784, 785
  Rhizopus, 785–787
zygote, 173, 784

PERCEPTIONS OF A RENEGADE MIND



DAVID ICKE


PERCEPTIONS
OF A
RENEGADE
MIND
DAVID ICKE

PERCEPTIONS OF A RENEGADE MIND

ickonic
publishing

First published in July 2021.

ickonic
publishing

New Enterprise House
St Helens Street
Derby
DE1 3GY
UK

email: gareth.icke@davidicke.com

Copyright © 2021 David Icke

No part of this book may be reproduced in any form without permission from the Publisher, except for the quotation of brief passages in criticism

Cover Design: Gareth Icke
Book Design: Neil Hague

British Library Cataloguing-in Publication Data
A catalogue record for this book is available from the British Library

eISBN 978-18384153-1-0

PERCEPTIONS OF A RENEGADE MIND

DAVID ICKE

Dedication:

To *Freeeeeedom!*

ICKONIC
THE ALTERNATIVE

NEW. DIFFERENT. REVOLUTIONARY

HUNDREDS OF CUTTING EDGE DOCUMENTARIES,
FEATURE FILMS, SERIES & PODCASTS.

SIGN UP NOW AT ICKONIC.COM

THE LIFE STORY OF DAVID ICKE

RENEGADE

THE FEATURE LENGTH FILM

AVAILABLE NOW AT DAVIDICKE.COM

Renegade:
Adjective
‘Having rejected tradition: Unconventional.’
Merriam-Webster Dictionary

Acquiescence to tyranny is the death of the spirit

You may be 38 years old, as I happen to be. And one day, some great opportunity stands before you and calls you to stand up for some great principle, some great issue, some great cause. And you refuse to do it because you are afraid … You refuse to do it because you want to live longer … You're afraid that you will lose your job, or you are afraid that you will be criticised or that you will lose your popularity, or you're afraid that somebody will stab you, or shoot at you or bomb your house; so you refuse to take the stand.

Well, you may go on and live until you are 90, but you're just as dead at 38 as you would be at 90. And the cessation of breathing in your life is but the belated announcement of an earlier death of the spirit.

Martin Luther King

How the few control the many and always have – the many do whatever they're told

'Forward, the Light Brigade!'
Was there a man dismayed?
Not though the soldier knew
Someone had blundered.
Theirs not to make reply,
Theirs not to reason why,
Theirs but to do and die.
Into the valley of Death
Rode the six hundred.

Cannon to right of them,
Cannon to left of them,
Cannon in front of them
Volleyed and thundered;
Stormed at with shot and shell,
Boldly they rode and well,
Into the jaws of Death,
Into the mouth of hell
Rode the six hundred

Alfred Lord Tennyson (1809-1892)

The mist is lifting slowly
I can see the way ahead
And I've left behind the empty streets
That once inspired my life
And the strength of the emotion
Is like thunder in the air
'Cos the promise that we made each other
Haunts me to the end

The secret of your beauty
And the mystery of your soul
I've been searching for in everyone I meet
And the times I've been mistaken
It's impossible to say
And the grass is growing
Underneath our feet

The words that I remember
From my childhood still are true
That there's none so blind
As those who will not see
And to those who lack the courage
And say it's dangerous to try
Well they just don't know
That love eternal will not be denied

I know you're out there somewhere
Somewhere, somewhere
I know you're out there somewhere

Somewhere you can hear my voice
I know I'll find you somehow
Somehow, somehow
I know I'll find you somehow
And somehow I'll return again to you

The Moody Blues

Are you a gutless wonder - or a Renegade Mind?

Monuments put from pen to paper,
Turns me into a gutless wonder,
And if you tolerate this,
Then your children will be next.
Gravity keeps my head down,
Or is it maybe shame ...

Manic Street Preachers

Rise like lions after slumber
In unvanquishable number.
Shake your chains to earth like dew
Which in sleep have fallen on you.
Ye are many – they are few.

Percy Shelley

Contents

CHAPTER 1 'I'm thinking' – Oh, but *are* you?
CHAPTER 2 Renegade perception
CHAPTER 3 The Pushbacker sting
CHAPTER 4 'Covid': The calculated catastrophe
CHAPTER 5 There *is no* 'virus'
CHAPTER 6 Sequence of deceit
CHAPTER 7 War on your mind
CHAPTER 8 'Reframing' insanity
CHAPTER 9 We must have it? So what is it?
CHAPTER 10 Human 2.0
CHAPTER 11 Who controls the Cult?
CHAPTER 12 Escaping Wetiko

POSTSCRIPT
APPENDIX Cowan-Kaufman-Morell Statement on Virus Isolation
BIBLIOGRAPHY
INDEX

CHAPTER ONE

I'm thinking' – Oh, but *are* you?

Think for yourself and let others enjoy the privilege of doing so too
Voltaire

French-born philosopher, mathematician and scientist René Descartes became famous for his statement in Latin in the 17th century which translates into English as: 'I think, therefore I am.'

On the face of it that is true. Thought reflects perception and perception leads to both behaviour and self-identity. In that sense 'we' are what we think. But who or what is doing the thinking and is thinking the only route to perception? Clearly, as we shall see, 'we' are not always the source of 'our' perception, indeed with regard to humanity as a whole this is rarely the case; and thinking is far from the only means of perception. Thought is the village idiot compared with other expressions of consciousness that we all have the potential to access and tap into. This has to be true when we *are* those other expressions of consciousness which are infinite in nature. We have forgotten this, or, more to the point, been manipulated to forget.

These are not just the esoteric musings of the navel. The whole foundation of human control and oppression is control of perception. Once perception is hijacked then so is behaviour which is dictated by perception. Collective perception becomes collective behaviour and collective behaviour is what we call human society. Perception is all and those behind human control know that which is

why perception is the target 24/7 of the psychopathic manipulators that I call the Global Cult. They know that if they dictate perception they will dictate behaviour and collectively dictate the nature of human society. They are further aware that perception is formed from information received and if they control the circulation of information they will to a vast extent direct human behaviour. Censorship of information and opinion has become globally Nazi-like in recent years and never more blatantly than since the illusory 'virus pandemic' was triggered out of China in 2019 and across the world in 2020. Why have billions submitted to house arrest and accepted fascistic societies in a way they would have never believed possible? Those controlling the information spewing from government, mainstream media and Silicon Valley (all controlled by the same Global Cult networks) told them they were in danger from a 'deadly virus' and only by submitting to house arrest and conceding their most basic of freedoms could they and their families be protected. This monumental and provable lie became the *perception* of the billions and therefore the *behaviour* of the billions. In those few words you have the whole structure and modus operandi of human control. Fear is a perception – **F**alse **E**motion **A**ppearing **R**eal – and fear is the currency of control. In short … get them by the balls (or give them the impression that you have) and their hearts and minds will follow. Nothing grips the dangly bits and freezes the rear-end more comprehensively than fear.

World number 1

There are two 'worlds' in what appears to be one 'world' and the prime difference between them is knowledge. First we have the mass of human society in which the population is maintained in coldly-calculated ignorance through control of information and the 'education' (indoctrination) system. That's all you really need to control to enslave billions in a perceptual delusion in which what are perceived to be *their* thoughts and opinions are ever-repeated mantras that the system has been downloading all their lives through 'education', media, science, medicine, politics and academia

in which the personnel and advocates are themselves overwhelmingly the perceptual products of the same repetition. Teachers and academics in general are processed by the same programming machine as everyone else, but unlike the great majority they never leave the 'education' program. It gripped them as students and continues to grip them as programmers of subsequent generations of students. The programmed become the programmers – the programmed programmers. The same can largely be said for scientists, doctors and politicians and not least because as the American writer Upton Sinclair said: 'It is difficult to get a man to understand something when his salary depends upon his not understanding it.' If your career and income depend on thinking the way the system demands then you will – bar a few free-minded exceptions – concede your mind to the Perceptual Mainframe that I call the Postage Stamp Consensus. This is a tiny band of perceived knowledge and possibility 'taught' (downloaded) in the schools and universities, pounded out by the mainstream media and on which all government policy is founded. Try thinking, and especially speaking and acting, outside of the 'box' of consensus and see what that does for your career in the Mainstream Everything which bullies, harasses, intimidates and ridicules the population into compliance. Here we have the simple structure which enslaves most of humanity in a perceptual prison cell for an entire lifetime and I'll go deeper into this process shortly. Most of what humanity is taught as fact is nothing more than programmed belief. American science fiction author Frank Herbert was right when he said: 'Belief can be manipulated. Only knowledge is dangerous.' In the 'Covid' age belief is promoted and knowledge is censored. It was always so, but never to the extreme of today.

World number 2

A 'number 2' is slang for 'doing a poo' and how appropriate that is when this other 'world' is doing just that on humanity every minute of every day. World number 2 is a global network of secret societies and semi-secret groups dictating the direction of society via

governments, corporations and authorities of every kind. I have spent more than 30 years uncovering and exposing this network that I call the Global Cult and knowing its agenda is what has made my books so accurate in predicting current and past events. Secret societies are secret for a reason. They want to keep their hoarded knowledge to themselves and their chosen initiates and to hide it from the population which they seek through ignorance to control and subdue. The whole foundation of the division between World 1 and World 2 is *knowledge*. What number 1 knows number 2 must not. Knowledge they have worked so hard to keep secret includes (a) the agenda to enslave humanity in a centrally-controlled global dictatorship, and (b) the nature of reality and life itself. The latter (b) must be suppressed to allow the former (a) to prevail as I shall be explaining. The way the Cult manipulates and interacts with the population can be likened to a spider's web. The 'spider' sits at the centre in the shadows and imposes its will through the web with each strand represented in World number 2 by a secret society, satanic or semi-secret group, and in World number 1 – the world of the seen – by governments, agencies of government, law enforcement, corporations, the banking system, media conglomerates and Silicon Valley (Fig 1 overleaf). The spider and the web connect and coordinate all these organisations to pursue the same global outcome while the population sees them as individual entities working randomly and independently. At the level of the web governments *are* the banking system *are* the corporations *are* the media *are* Silicon Valley *are* the World Health Organization working from their inner cores as one unit. Apparently unconnected countries, corporations, institutions, organisations and people are on the *same team* pursuing the same global outcome. Strands in the web immediately around the spider are the most secretive and exclusive secret societies and their membership is emphatically restricted to the Cult inner-circle emerging through the generations from particular bloodlines for reasons I will come to. At the core of the core you would get them in a single room. That's how many people are dictating the direction of human society and its transformation

through the ‘Covid’ hoax and other means. As the web expands out from the spider we meet the secret societies that many people will be aware of – the Freemasons, Knights Templar, Knights of Malta, Opus Dei, the inner sanctum of the Jesuit Order, and such like. Note how many are connected to the Church of Rome and there is a reason for that. The Roman Church was established as a revamp, a rebranding, of the relocated ‘Church’ of Babylon and the Cult imposing global tyranny today can be tracked back to Babylon and Sumer in what is now Iraq.

Figure 1: The global web through which the few control the many. (Image Neil Hague.)

Inner levels of the web operate in the unseen away from the public eye and then we have what I call the cusp organisations located at the point where the hidden meets the seen. They include a series of satellite organisations answering to a secret society founded in London in the late 19th century called the Round Table and among them are the Royal Institute of International Affairs (UK, founded in 1920); Council on Foreign Relations (US, 1921); Bilderberg Group (worldwide, 1954); Trilateral Commission (US/worldwide, 1972); and the Club of Rome (worldwide, 1968) which was created to exploit environmental concerns to justify the centralisation of global power to ‘save the planet’. The Club of Rome instigated with others the human-caused climate change hoax which has led to all the ‘green

new deals' demanding that very centralisation of control. Cusp organisations, which include endless 'think tanks' all over the world, are designed to coordinate a single global policy between political and business leaders, intelligence personnel, media organisations and anyone who can influence the direction of policy in their own sphere of operation. Major players and regular attenders will know what is happening – or some of it – while others come and go and are kept overwhelmingly in the dark about the big picture. I refer to these cusp groupings as semi-secret in that they can be publicly identified, but what goes on at the inner-core is kept very much 'in house' even from most of their members and participants through a fiercely-imposed system of compartmentalisation. Only let them know what they need to know to serve your interests and no more. The structure of secret societies serves as a perfect example of this principle. Most Freemasons never get higher than the bottom three levels of 'degree' (degree of knowledge) when there are 33 official degrees of the Scottish Rite. Initiates only qualify for the next higher 'compartment' or degree if those at that level choose to allow them. Knowledge can be carefully assigned only to those considered 'safe'. I went to my local Freemason's lodge a few years ago when they were having an 'open day' to show how cuddly they were and when I chatted to some of them I was astonished at how little the rank and file knew even about the most ubiquitous symbols they use. The mushroom technique – keep them in the dark and feed them bullshit – applies to most people in the web as well as the population as a whole. Sub-divisions of the web mirror in theme and structure transnational corporations which have a headquarters somewhere in the world dictating to all their subsidiaries in different countries. Subsidiaries operate in their methodology and branding to the same centrally-dictated plan and policy in pursuit of particular ends. The Cult web functions in the same way. Each country has its own web as a subsidiary of the global one. They consist of networks of secret societies, semi-secret groups and bloodline families and their job is to impose the will of the spider and the global web in their particular country. Subsidiary networks control and manipulate the national political system, finance, corporations, media, medicine, etc. to

ensure that they follow the globally-dictated Cult agenda. These networks were the means through which the 'Covid' hoax could be played out with almost every country responding in the same way.

The 'Yessir' pyramid

Compartmentalisation is the key to understanding how a tiny few can dictate the lives of billions when combined with a top-down sequence of imposition and acquiescence. The inner core of the Cult sits at the peak of the pyramidal hierarchy of human society (Fig 2 overleaf). It imposes its will – its agenda for the world – on the level immediately below which acquiesces to that imposition. This level then imposes the Cult will on the level below them which acquiesces and imposes on the next level. Very quickly we meet levels in the hierarchy that have no idea there even is a Cult, but the sequence of imposition and acquiescence continues down the pyramid in just the same way. 'I don't know why we are doing this but the order came from "on-high" and so we better just do it.' Alfred Lord Tennyson said of the cannon fodder levels in his poem *The Charge of the Light Brigade*: 'Theirs not to reason why; theirs but to do and die.' The next line says that 'into the valley of death rode the six hundred' and they died because they obeyed without question what their perceived 'superiors' told them to do. In the same way the population capitulated to 'Covid'. The whole hierarchical pyramid functions like this to allow the very few to direct the enormous many. Eventually imposition-acquiescence-imposition-acquiescence comes down to the mass of the population at the foot of the pyramid. If they acquiesce to those levels of the hierarchy imposing on them (governments/law enforcement/doctors/media) a circuit is completed between the population and the handful of super-psychopaths in the Cult inner core at the top of the pyramid. Without a circuit-breaking refusal to obey, the sequence of imposition and acquiescence allows a staggeringly few people to impose their will upon the entirety of humankind. We are looking at the very sequence that has subjugated billions since the start of 2020. Our freedom has not been taken from us. Humanity has given it

away. Fascists do not impose fascism because there are not enough of them. Fascism is imposed by the population acquiescing to fascism. Put another way allowing their perceptions to be programmed to the extent that leads to the population giving their freedom away by giving their perceptions – their mind – away. If this circuit is not broken by humanity ceasing to cooperate with their own enslavement then nothing can change. For that to happen people have to critically think and see through the lies and window dressing and then summon the backbone to act upon what they see. The Cult spends its days working to stop either happening and its methodology is systematic and highly detailed, but it can be overcome and that is what this book is all about.

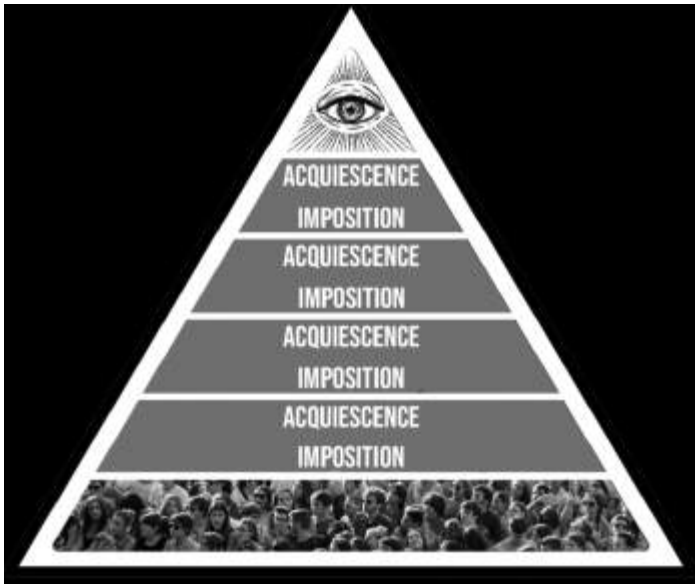


Figure 2: The simple sequence of imposition and compliance that allows a handful of people at the peak of the pyramid to dictate the lives of billions.

The Life Program

Okay, back to world number 1 or the world of the 'masses'. Observe the process of what we call 'life' and it is a perceptual download from cradle to grave. The Cult has created a global structure in which perception can be programmed and the program continually topped-up with what appears to be constant confirmation that the program is indeed true reality. The important word here is 'appears'.

This is the structure, the fly-trap, the Postage Stamp Consensus or Perceptual Mainframe, which represents that incredibly narrow band of perceived possibility delivered by the 'education' system, mainstream media, science and medicine. From the earliest age the download begins with parents who have themselves succumbed to the very programming their children are about to go through. Most parents don't do this out of malevolence and mostly it is quite the opposite. They do what they believe is best for their children and that is what the program has told them is best. Within three or four years comes the major transition from parental programming to full-blown state (Cult) programming in school, college and university where perceptually-programmed teachers and academics pass on their programming to the next generations. Teachers who resist are soon marginalised and their careers ended while children who resist are called a problem child for whom Ritalin may need to be prescribed. A few years after entering the 'world' children are under the control of authority figures representing the state telling them when they have to be there, when they can leave and when they can speak, eat, even go to the toilet. This is calculated preparation for a lifetime of obeying authority in all its forms. Reflex-action fear of authority is instilled by authority from the start. Children soon learn the carrot and stick consequences of obeying or defying authority which is underpinned daily for the rest of their life. Fortunately I daydreamed through this crap and never obeyed authority simply because it told me to. This approach to my alleged 'betters' continues to this day. There can be consequences of pursuing open-minded freedom in a world of closed-minded conformity. I spent a lot of time in school corridors after being ejected from the classroom for not taking some of it seriously and now I spend a lot of time being ejected from Facebook, YouTube and Twitter. But I can tell you that being true to yourself and not compromising your self-respect is far more exhilarating than bowing to authority for authority's sake. You don't have to be a sheep to the shepherd (authority) and the sheep dog (fear of not obeying authority).

The perceptual download continues throughout the formative years in school, college and university while script-reading 'teachers', 'academics' 'scientists', 'doctors' and 'journalists' insist that ongoing generations must be as programmed as they are. Accept the program or you will not pass your 'exams' which confirm your 'degree' of programming. It is tragic to think that many parents pressure their offspring to work hard at school to download the program and qualify for the next stage at college and university. The late, great, American comedian George Carlin said: 'Here's a bumper sticker I'd like to see: We are proud parents of a child who has resisted his teachers' attempts to break his spirit and bend him to the will of his corporate masters.' Well, the best of luck finding many of those, George. Then comes the moment to leave the formal programming years in academia and enter the 'adult' world of work. There you meet others in your chosen or prescribed arena who went through the same Postage Stamp Consensus program before you did. There is therefore overwhelming agreement between almost everyone on the basic foundations of Postage Stamp reality and the rejection, even contempt, of the few who have a mind of their own and are prepared to use it. This has two major effects. Firstly, the consensus confirms to the programmed that their download is really how things are. I mean, everyone knows that, right? Secondly, the arrogance and ignorance of Postage Stamp adherents ensure that anyone questioning the program will have unpleasant consequences for seeking their own truth and not picking their perceptions from the shelf marked: 'Things you must believe without question and if you don't you're a dangerous lunatic conspiracy theorist and a harebrained nutter'.

Every government, agency and corporation is founded on the same Postage Stamp prison cell and you can see why so many people believe the same thing while calling it their own 'opinion'. Fusion of governments and corporations in pursuit of the same agenda was the definition of fascism described by Italian dictator Benito Mussolini. The pressure to conform to perceptual norms downloaded for a lifetime is incessant and infiltrates society right

down to family groups that become censors and condemners of their own 'black sheep' for not, ironically, being sheep. We have seen an explosion of that in the 'Covid' era. Cult-owned global media unleashes its propaganda all day every day in support of the Postage Stamp and targets with abuse and ridicule anyone in the public eye who won't bend their mind to the will of the tyranny. Any response to this is denied (certainly in my case). They don't want to give a platform to expose official lies. Cult-owned-and-created Internet giants like Facebook, Google, YouTube and Twitter delete you for having an unapproved opinion. Facebook boasts that its AI censors delete 97-percent of 'hate speech' before anyone even reports it. Much of that 'hate speech' will simply be an opinion that Facebook and its masters don't want people to see. Such perceptual oppression is widely known as fascism. Even Facebook executive Benny Thomas, a 'CEO Global Planning Lead', said in comments secretly recorded by investigative journalism operation Project Veritas that Facebook is 'too powerful' and should be broken up:

I mean, no king in history has been the ruler of two billion people, but Mark Zuckerberg is … And he's 36. That's too much for a 36-year-old ... You should not have power over two billion people. I just think that's wrong.

Thomas said Facebook-owned platforms like Instagram, Oculus, and WhatsApp needed to be separate companies. 'It's too much power when they're all one together'. That's the way the Cult likes it, however. We have an executive of a Cult organisation in Benny Thomas that doesn't know there is a Cult such is the compartmentalisation. Thomas said that Facebook and Google 'are no longer companies, they're countries'. Actually they are more powerful than countries on the basis that if you control information you control perception and control human society.

I love my oppressor

Another expression of this psychological trickery is for those who realise they are being pressured into compliance to eventually

convince themselves to believe the official narratives to protect their self-respect from accepting the truth that they have succumbed to meek and subservient compliance. Such people become some of the most vehement defenders of the system. You can see them everywhere screaming abuse at those who prefer to think for themselves and by doing so reminding the compliers of their own capitulation to conformity. 'You are talking dangerous nonsense you Covidiot!!' Are you trying to convince me or yourself? It is a potent form of Stockholm syndrome which is defined as: 'A psychological condition that occurs when a victim of abuse identifies and attaches, or bonds, positively with their abuser.' An example is hostages bonding and even 'falling in love' with their kidnappers. The syndrome has been observed in domestic violence, abused children, concentration camp inmates, prisoners of war and many and various Satanic cults. These are some traits of Stockholm syndrome listed at goodtherapy.org:

- Positive regard towards perpetrators of abuse or captor [see 'Covid'].
- Failure to cooperate with police and other government authorities when it comes to holding perpetrators of abuse or kidnapping accountable [or in the case of 'Covid' cooperating with the police to enforce and defend their captors' demands].
- Little or no effort to escape [see 'Covid'].
- Belief in the goodness of the perpetrators or kidnappers [see 'Covid'].
- Appeasement of captors. This is a manipulative strategy for maintaining one's safety. As victims get rewarded – perhaps with less abuse or even with life itself – their appeasing behaviours are reinforced [see 'Covid'].
- Learned helplessness. This can be akin to 'if you can't beat 'em, join 'em'. As the victims fail to escape the abuse or captivity, they may start giving up and soon realize it's just easier for everyone if they acquiesce all their power to their captors [see 'Covid'].

- Feelings of pity toward the abusers, believing they are actually victims themselves. Because of this, victims may go on a crusade or mission to 'save' [protect] their abuser [see the venom unleashed on those challenging the official 'Covid' narrative].
- Unwillingness to learn to detach from their perpetrators and heal. In essence, victims may tend to be less loyal to themselves than to their abuser [*definitely* see 'Covid'].

Ponder on those traits and compare them with the behaviour of great swathes of the global population who have defended governments and authorities which have spent every minute destroying their lives and livelihoods and those of their children and grandchildren since early 2020 with fascistic lockdowns, house arrest and employment deletion to 'protect' them from a 'deadly virus' that their abusers' perceptually created to bring about this very outcome. We are looking at mass Stockholm syndrome. All those that agree to concede their freedom will believe those perceptions are originating in their own independent 'mind' when in fact by conceding their reality to Stockholm syndrome they have by definition conceded any independence of mind. Listen to the 'opinions' of the acquiescing masses in this 'Covid' era and what gushes forth is the repetition of the official version of everything delivered unprocessed, unfiltered and unquestioned. The whole programming dynamic works this way. I must be free because I'm told that I am and so I think that I am.

You can see what I mean with the chapter theme of 'I'm thinking – Oh, but *are* you?' The great majority are not thinking, let alone for themselves. They are repeating what authority has told them to believe which allows them to be controlled. Weaving through this mentality is the fear that the 'conspiracy theorists' are right and this again explains the often hysterical abuse that ensues when you dare to contest the official narrative of anything. Denial is the mechanism of hiding from yourself what you don't want to be true. Telling people what they want to hear is easy, but it's an infinitely greater challenge to tell them what they would rather not be happening.

One is akin to pushing against an open door while the other is met with vehement resistance no matter what the scale of evidence. I don't want it to be true so I'll convince myself that it's not. Examples are everywhere from the denial that a partner is cheating despite all the signs to the reflex-action rejection of any idea that world events in which country after country act in exactly the same way are centrally coordinated. To accept the latter is to accept that a force of unspeakable evil is working to destroy your life and the lives of your children with nothing too horrific to achieve that end. Who the heck wants that to be true? But if we don't face reality the end is duly achieved and the consequences are far worse and ongoing than breaking through the walls of denial today with the courage to make a stand against tyranny.

Connect the dots – but how?

A crucial aspect of perceptual programming is to portray a world in which everything is random and almost nothing is connected to anything else. Randomness cannot be coordinated by its very nature and once you perceive events as random the idea they could be connected is waved away as the rantings of the tinfoil-hat brigade. You can't plan and coordinate random you idiot! No, you can't, but you can hide the coldly-calculated and long-planned behind the *illusion* of randomness. A foundation manifestation of the Renegade Mind is to scan reality for patterns that connect the apparently random and turn pixels and dots into pictures. This is the way I work and have done so for more than 30 years. You look for similarities in people, modus operandi and desired outcomes and slowly, then ever quicker, the picture forms. For instance: There would seem to be no connection between the 'Covid pandemic' hoax and the human-caused global-warming hoax and yet they are masks (appropriately) on the same face seeking the same outcome. Those pushing the global warming myth through the Club of Rome and other Cult agencies are driving the lies about 'Covid' – Bill Gates is an obvious one, but they are endless. Why would the same people be involved in both when they are clearly not connected? Oh, but they

are. Common themes with personnel are matched by common goals. The 'solutions' to both 'problems' are centralisation of global power to impose the will of the few on the many to 'save' humanity from 'Covid' and save the planet from an 'existential threat' (we need 'zero Covid' and 'zero carbon emissions'). These, in turn, connect with the 'dot' of globalisation which was coined to describe the centralisation of global power in every area of life through incessant political and corporate expansion, trading blocks and superstates like the European Union. If you are the few and you want to control the many you have to centralise power and decision-making. The more you centralise power the more power the few at the centre will have over the many; and the more that power is centralised the more power those at the centre have to centralise even quicker. The momentum of centralisation gets faster and faster which is exactly the process we have witnessed. In this way the hoaxed 'pandemic' and the fakery of human-caused global warming serve the interests of globalisation and the seizure of global power in the hands of the Cult inner-circle which is behind 'Covid', 'climate change' *and* globalisation. At this point random 'dots' become a clear and obvious picture or pattern.

Klaus Schwab, the classic Bond villain who founded the Cult's Gates-funded World Economic Forum, published a book in 2020, *The Great Reset*, in which he used the 'problem' of 'Covid' to justify a total transformation of human society to 'save' humanity from 'climate change'. Schwab said: 'The pandemic represents a rare but narrow window of opportunity to reflect, reimagine, and reset our world.' What he didn't mention is that the Cult he serves is behind both hoaxes as I show in my book *The Answer*. He and the Cult don't have to reimagine the world. They know precisely what they want and that's why they destroyed human society with 'Covid' to 'build back better' in their grand design. Their job is not to imagine, but to get humanity to imagine and agree with their plans while believing it's all random. It must be pure coincidence that 'The Great Reset' has long been the Cult's code name for the global imposition of fascism and replaced previous code-names of the 'New World

Order' used by Cult frontmen like Father George Bush and the 'New Order of the Ages' which emerged from Freemasonry and much older secret societies. New Order of the Ages appears on the reverse of the Great Seal of the United States as 'Novus ordo seclorum' underneath the Cult symbol used since way back of the pyramid and all seeing-eye (Fig 3). The pyramid is the hierarchy of human control headed by the illuminated eye that symbolises the force behind the Cult which I will expose in later chapters. The term 'Annuit Coeptis' translates as 'He favours our undertaking'. We are told the 'He' is the Christian god, but 'He' is not as I will be explaining.

Figure 3: The all-seeing eye of the Cult 'god' on the Freemason-designed Great Seal of the United States and also on the dollar bill.

Having you on

Two major Cult techniques of perceptual manipulation that relate to all this are what I have called since the 1990s Problem-Reaction-Solution (PRS) and the Totalitarian Tiptoe (TT). They can be uncovered by the inquiring mind with a simple question: Who benefits? The answer usually identifies the perpetrators of a given action or happening through the concept of 'he who most benefits from a crime is the one most likely to have committed it'. The Latin 'Cue bono?' – Who benefits? – is widely attributed to the Roman orator and statesman Marcus Tullius Cicero. No wonder it goes back so far when the concept has been relevant to human behaviour since

history was recorded. Problem-Reaction-Solution is the technique used to manipulate us every day by covertly creating a problem (or the illusion of one) and offering the solution to the problem (or the illusion of one). In the first phase you create the problem and blame someone or something else for why it has happened. This may relate to a financial collapse, terrorist attack, war, global warming or pandemic, anything in fact that will allow you to impose the 'solution' to change society in the way you desire at that time. The 'problem' doesn't have to be real. PRS is manipulation of perception and all you need is the population to believe the problem is real. Human-caused global warming and the 'Covid pandemic' only have to be *perceived* to be real for the population to accept the 'solutions' of authority. I refer to this technique as NO-Problem-Reaction-Solution. Billions did not meekly accept house arrest from early 2020 because there was a real deadly 'Covid pandemic' but because they perceived – believed – that to be the case. The antidote to Problem-Reaction-Solution is to ask who benefits from the proposed solution. Invariably it will be anyone who wants to justify more control through deletion of freedom and centralisation of power and decision-making.

The two world wars were Problem-Reaction-Solutions that transformed and realigned global society. Both were manipulated into being by the Cult as I have detailed in books since the mid-1990s. They dramatically centralised global power, especially World War Two, which led to the United Nations and other global bodies thanks to the overt and covert manipulations of the Rockefeller family and other Cult bloodlines like the Rothschilds. The UN is a stalking horse for full-blown world government that I will come to shortly. The land on which the UN building stands in New York was donated by the Rockefellers and the same Cult family was behind Big Pharma scalpel and drug 'medicine' and the creation of the World Health Organization as part of the UN. They have been stalwarts of the eugenics movement and funded Hitler's race-purity expert' Ernst Rudin. The human-caused global warming hoax has been orchestrated by the Club of Rome through the UN which is

manufacturing both the 'problem' through its Intergovernmental Panel on Climate Change and imposing the 'solution' through its Agenda 21 and Agenda 2030 which demand the total centralisation of global power to 'save the world' from a climate hoax the United Nations is itself perpetrating. What a small world the Cult can be seen to be particularly among the inner circles. The bedfellow of Problem-Reaction-Solution is the Totalitarian Tiptoe which became the Totalitarian Sprint in 2020. The technique is fashioned to hide the carefully-coordinated behind the cover of apparently random events. You start the sequence at 'A' and you know you are heading for 'Z'. You don't want people to know that and each step on the journey is presented as a random happening while all the steps strung together lead in the same direction. The speed may have quickened dramatically in recent times, but you can still see the incremental approach of the Tiptoe in the case of 'Covid' as each new imposition takes us deeper into fascism. Tell people they have to do this or that to get back to 'normal', then this and this and this. With each new demand adding to the ones that went before the population's freedom is deleted until it disappears. The spider wraps its web around the flies more comprehensively with each new diktat. I'll highlight this in more detail when I get to the 'Covid' hoax and how it has been pulled off. Another prime example of the Totalitarian Tiptoe is how the Cult-created European Union went from a 'free-trade zone' to a centralised bureaucratic dictatorship through the Tiptoe of incremental centralisation of power until nations became mere administrative units for Cult-owned dark suits in Brussels.

The antidote to ignorance is knowledge which the Cult seeks vehemently to deny us, but despite the systematic censorship to that end the Renegade Mind can overcome this by vociferously seeking out the facts no matter the impediments put in the way. There is also a method of thinking and perceiving – *knowing* – that doesn't even need names, dates, place-type facts to identify the patterns that reveal the story. I'll get to that in the final chapter. All you need to know about the manipulation of human society and to what end is still out there – *at the time of writing* – in the form of books, videos

and websites for those that really want to breach the walls of programmed perception. To access this knowledge requires the abandonment of the mainstream media as a source of information in the awareness that this is owned and controlled by the Cult and therefore promotes mass perceptions that suit the Cult. Mainstream media lies all day, every day. That is its function and very reason for being. Where it does tell the truth, here and there, is only because the truth and the Cult agenda very occasionally coincide. If you look for fact and insight to the BBC, CNN and virtually all the rest of them you are asking to be conned and perceptually programmed.

Know the outcome and you'll see the journey

Events seem random when you have no idea where the world is being taken. Once you do the random becomes the carefully planned. Know the outcome and you'll see the journey is a phrase I have been using for a long time to give context to daily happenings that appear unconnected. Does a problem, or illusion of a problem, trigger a proposed 'solution' that further drives society in the direction of the outcome? Invariably the answer will be yes and the random – *abracadabra* – becomes the clearly coordinated. So what is this outcome that unlocks the door to a massively expanded understanding of daily events? I will summarise its major aspects – the fine detail is in my other books – and those new to this information will see that the world they thought they were living in is a very different place. The foundation of the Cult agenda is the incessant centralisation of power and all such centralisation is ultimately in pursuit of Cult control on a global level. I have described for a long time the planned world structure of top-down dictatorship as the Hunger Games Society. The term obviously comes from the movie series which portrayed a world in which a few living in military-protected hi-tech luxury were the overlords of a population condemned to abject poverty in isolated 'sectors' that were not allowed to interact. 'Covid' lockdowns and travel bans anyone? The 'Hunger Games' pyramid of structural control has the inner circle of the Cult at the top with pretty much the entire

population at the bottom under their control through dependency for survival on the Cult. The whole structure is planned to be protected and enforced by a military-police state (Fig 4).

Here you have the reason for the global lockdowns of the fake pandemic to coldly destroy independent incomes and livelihoods and make everyone dependent on the 'state' (the Cult that controls the 'states'). I have warned in my books for many years about the plan to introduce a 'guaranteed income' – a barely survivable pittance – designed to impose dependency when employment was destroyed by AI technology and now even more comprehensively at great speed by the 'Covid' scam. Once the pandemic was played and lockdown consequences began to delete independent income the authorities began to talk right on cue about the need for a guaranteed income and a 'Great Reset'. Guaranteed income will be presented as benevolent governments seeking to help a desperate people – desperate as a direct result of actions of the same governments. The truth is that such payments are a trap. You will only get them if you do exactly what the authorities demand including mass vaccination (genetic manipulation). We have seen this theme already in Australia where those dependent on government benefits have them reduced if parents don't agree to have their children vaccinated according to an insane health-destroying government-dictated schedule. Calculated economic collapse applies to governments as well as people. The Cult wants rid of countries through the creation of a world state with countries broken up into regions ruled by a world government and super states like the European Union. Countries must be bankrupted, too, to this end and it's being achieved by the trillions in 'rescue packages' and furlough payments, trillions in lost taxation, and money-no-object spending on 'Covid' including constant all-medium advertising (programming) which has made the media dependent on government for much of its income. The day of reckoning is coming – as planned – for government spending and given that it has been made possible by printing money and not by production/taxation there is inflation on the way that has the

potential to wipe out monetary value. In that case there will be no need for the Cult to steal your money. It just won't be worth anything (see the German Weimar Republic before the Nazis took over). Many have been okay with lockdowns while getting a percentage of their income from so-called furlough payments without having to work. Those payments are dependent, however, on people having at least a theoretical job with a business considered non-essential and ordered to close. As these business go under because they are closed by lockdown after lockdown the furlough stops and it will for everyone eventually. Then what? The 'then what?' is precisely the idea.

THE COVID-19 CREATED HUNGER GAMES SOCIETY
THE ELITE - 1%
THE GLOBAL POLICE STATE
MANDATORY VACCINATION - ID - SOCIAL CREDIT - 666
CLOSED
FOOD BANK
THE MASSES LIVING IN POVERTY

Figure 4: The Hunger Games Society structure I have long warned was planned and now the 'Covid' hoax has made it possible. This is the real reason for lockdowns.

Hired hands

Between the Hunger Games Cult elite and the dependent population is planned to be a vicious military-police state (a fusion of the two into one force). This has been in the making for a long time with police looking ever more like the military and carrying weapons to match. The pandemic scam has seen this process accelerate so fast as

lockdown house arrest is brutally enforced by carefully recruited fascist minds and gormless system-servers. The police and military are planned to merge into a centrally-directed world army in a global structure headed by a world government which wouldn't be elected even by the election fixes now in place. The world army is not planned even to be human and instead wars would be fought, primarily against the population, using robot technology controlled by artificial intelligence. I have been warning about this for decades and now militaries around the world are being transformed by this very AI technology. The global regime that I describe is a particular form of fascism known as a technocracy in which decisions are not made by clueless and co-opted politicians but by unelected technocrats – scientists, engineers, technologists and bureaucrats. Cult-owned-and-controlled Silicon Valley giants are examples of technocracy and they already have far more power to direct world events than governments. They are with their censorship *selecting* governments. I know that some are calling the 'Great Reset' a Marxist communist takeover, but fascism and Marxism are different labels for the same tyranny. Tell those who lived in fascist Germany and Stalinist Russia that there was a difference in the way their freedom was deleted and their lives controlled. I could call it a fascist technocracy or a Marxist technocracy and they would be equally accurate. The Hunger Games society with its world government structure would oversee a world army, world central bank and single world cashless currency imposing its will on a microchipped population (Fig 5). Scan its different elements and see how the illusory pandemic is forcing society in this very direction at great speed. Leaders of 23 countries and the World Health Organization (WHO) backed the idea in March, 2021, of a global treaty for 'international cooperation' in 'health emergencies' and nations should 'come together as a global community for peaceful cooperation that extends beyond this crisis'. Cut the Orwellian bullshit and this means another step towards global government. The plan includes a cashless digital money system that I first warned about in 1993. Right at the start of 'Covid' the deeply corrupt Tedros

Adhanom Ghebreyesus, the crooked and merely gofer 'head' of the World Health Organization, said it was possible to catch the 'virus' by touching cash and it was better to use cashless means. The claim was ridiculous nonsense and like the whole 'Covid' mind-trick it was nothing to do with 'health' and everything to do with pushing every aspect of the Cult agenda. As a result of the Tedros lie the use of cash has plummeted. The Cult script involves a single world digital currency that would eventually be technologically embedded in the body. China is a massive global centre for the Cult and if you watch what is happening there you will know what is planned for everywhere. The Chinese government is developing a digital currency which would allow fines to be deducted immediately via AI for anyone caught on camera breaking its fantastic list of laws and the money is going to be programmable with an expiry date to ensure that no one can accrue wealth except the Cult and its operatives.

WORLD GOVERNMENT
UNELECTED - RUN BY TECHNOCRATS
WORLD ARMY • CENTRAL BANK • CURRENCY •
HUMANS CONNECTED TO A.I.
SUPERSTATES LIKE THE EU AND CENTRALLY
CONTROLLED 'TRADING BLOCS'
COUNTRIES REPLACED BY POWERLESS REGIONS
GLOBAL MONOCULTURE
THE PEOPLE

Figure 5: The structure of global control the Cult has been working towards for so long and this has been enormously advanced by the 'Covid' illusion.

Serfdom is so smart

The Cult plan is far wider, extreme, and more comprehensive than even most conspiracy researchers appreciate and I will come to the true depths of deceit and control in the chapters 'Who controls the

Cult?' and 'Escaping Wetiko'. Even the world that we know is crazy enough. We are being deluged with ever more sophisticated and controlling technology under the heading of 'smart'. We have smart televisions, smart meters, smart cards, smart cars, smart driving, smart roads, smart pills, smart patches, smart watches, smart skin, smart borders, smart pavements, smart streets, smart cities, smart communities, smart environments, smart growth, smart planet ... smart *everything* around us. Smart technologies and methods of operation are designed to interlock to create a global Smart Grid connecting the entirety of human society including human minds to create a centrally-dictated 'hive' mind. 'Smart cities' is code for densely-occupied megacities of total surveillance and control through AI. Ever more destructive frequency communication systems like 5G have been rolled out without any official testing for health and psychological effects (colossal). 5G/6G/7G systems are needed to run the Smart Grid and each one becomes more destructive of body and mind. Deleting independent income is crucial to forcing people into these AI-policed prisons by ending private property ownership (except for the Cult elite). The Cult's Great Reset now openly foresees a global society in which no one will own any possessions and everything will be rented while the Cult would own literally everything under the guise of government and corporations. The aim has been to use the lockdowns to destroy sources of income on a mass scale and when the people are destitute and in unrepayable amounts of debt (problem) Cult assets come forward with the pledge to write-off debt in return for handing over all property and possessions (solution). Everything – literally everything including people – would be connected to the Internet via AI. I was warning years ago about the coming Internet of Things (IoT) in which all devices and technology from your car to your fridge would be plugged into the Internet and controlled by AI. Now we are already there with much more to come. The next stage is the Internet of Everything (IoE) which is planned to include the connection of AI to the human brain and body to replace the human mind with a centrally-controlled AI mind. Instead of perceptions

being manipulated through control of information and censorship those perceptions would come direct from the Cult through AI. What do you think? You think whatever AI decides that you think. In human terms there would be no individual 'think' any longer. Too incredible? The ravings of a lunatic? Not at all. Cult-owned crazies in Silicon Valley have been telling us the plan for years without explaining the real motivation and calculated implications. These include Google executive and 'futurist' Ray Kurzweil who highlights the year 2030 for when this would be underway. He said:

Our thinking ... will be a hybrid of biological and non-biological thinking ... humans will be able to extend their limitations and 'think in the cloud' ... We're going to put gateways to the cloud in our brains ... We're going to gradually merge and enhance ourselves ... In my view, that's the nature of being human – we transcend our limitations.

As the technology becomes vastly superior to what we are then the small proportion that is still human gets smaller and smaller and smaller until it's just utterly negligible.

The sales-pitch of Kurzweil and Cult-owned Silicon Valley is that this would make us 'super-human' when the real aim is to make us post-human and no longer 'human' in the sense that we have come to know. The entire global population would be connected to AI and become the centrally-controlled 'hive-mind' of externally-delivered perceptions. The Smart Grid being installed to impose the Cult's will on the world is being constructed to allow particular locations – even one location – to control the whole global system. From these prime control centres, which absolutely include China and Israel, anything connected to the Internet would be switched on or off and manipulated at will. Energy systems could be cut, communication via the Internet taken down, computer-controlled driverless autonomous vehicles driven off the road, medical devices switched off, the potential is limitless given how much AI and Internet connections now run human society. We have seen nothing yet if we allow this to continue. Autonomous vehicle makers are working with law enforcement to produce cars designed to automatically pull over if they detect a police or emergency vehicle flashing from up to 100 feet away. At a police stop the car would be unlocked and the

window rolled down automatically. Vehicles would only take you where the computer (the state) allowed. The end of petrol vehicles and speed limiters on all new cars in the UK and EU from 2022 are steps leading to electric computerised transport over which ultimately you have no control. The picture is far bigger even than the Cult global network or web and that will become clear when I get to the nature of the 'spider'. There is a connection between all these happenings and the instigation of DNA-manipulating 'vaccines' (which aren't 'vaccines') justified by the 'Covid' hoax. That connection is the unfolding plan to transform the human body from a biological to a synthetic biological state and this is why synthetic biology is such a fast-emerging discipline of mainstream science. 'Covid vaccines' are infusing self-replicating synthetic genetic material into the cells to cumulatively take us on the Totalitarian Tiptoe from Human 1.0 to the synthetic biological Human 2.0 which will be physically and perceptually attached to the Smart Grid to one hundred percent control every thought, perception and deed. Humanity needs to wake up and *fast*.

This is the barest explanation of where the 'outcome' is planned to go but it's enough to see the journey happening all around us. Those new to this information will already see 'Covid' in a whole new context. I will add much more detail as we go along, but for the minutiae evidence see my mega-works, *The Answer, The Trigger* and *Everything You Need to Know But Have Never Been Told*.

Now – how does a Renegade Mind see the 'world'?

CHAPTER TWO

Renegade Perception

It is one thing to be clever and another to be wise
George R.R. Martin

A simple definition of the difference between a programmed mind and a Renegade Mind would be that one sees only dots while the other connects them to see the picture. Reading reality with accuracy requires the observer to (a) know the planned outcome and (b) realise that everything, but *everything*, is connected.

The entirety of infinite reality is connected – that's its very nature – and with human society an expression of infinite reality the same must apply. Simple cause and effect is a connection. The effect is triggered by the cause and the effect then becomes the cause of another effect. Nothing happens in isolation because it *can't*. Life in whatever reality is simple choice and consequence. We make choices and these lead to consequences. If we don't like the consequences we can make different choices and get different consequences which lead to other choices and consequences. The choice and the consequence are not only connected they are indivisible. You can't have one without the other as an old song goes. A few cannot control the world unless those being controlled allow that to happen – cause and effect, choice and consequence. Control – who has it and who doesn't – is a two-way process, a symbiotic relationship, involving the controller and controlled. 'They took my freedom away!!' Well, yes, but you also gave it to them. Humanity is

subjected to mass control because humanity has acquiesced to that control. This is all cause and effect and literally a case of give and take. In the same way world events of every kind are connected and the Cult works incessantly to sell the illusion of the random and coincidental to maintain the essential (to them) perception of dots that hide the picture. Renegade Minds know this and constantly scan the world for patterns of connection. This is absolutely pivotal in understanding the happenings in the world and without that perspective clarity is impossible. First you know the planned outcome and then you identify the steps on the journey – the day-by-day apparently random which, when connected in relation to the outcome, no longer appear as individual events, but as the proverbial *chain* of events leading in the same direction. I'll give you some examples:

Political puppet show

We are told to believe that politics is 'adversarial' in that different parties with different beliefs engage in an endless tussle for power. There may have been some truth in that up to a point – and only a point – but today divisions between 'different' parties are rhetorical not ideological. Even the rhetorical is fusing into one-speak as the parties eject any remaining free thinkers while others succumb to the ever-gathering intimidation of anyone with the 'wrong' opinion. The Cult is not a new phenomenon and can be traced back thousands of years as my books have documented. Its intergenerational initiates have been manipulating events with increasing effect the more that global power has been centralised. In ancient times the Cult secured control through the system of monarchy in which 'special' bloodlines (of which more later) demanded the right to rule as kings and queens simply by birthright and by vanquishing others who claimed the same birthright. There came a time, however, when people had matured enough to see the unfairness of such tyranny and demanded a say in who governed them. Note the word – *governed* them. Not served them – *governed* them, hence government defined as 'the political direction and control exercised over the

actions of the members, citizens, or inhabitants of communities, societies, and states; direction of the affairs of a state, community, etc.' Governments exercise control over rather than serve just like the monarchies before them. Bizarrely there are still countries like the United Kingdom which are ruled by a monarch *and* a government that officially answers to the monarch. The UK head of state and that of Commonwealth countries such as Canada, Australia and New Zealand is 'selected' by who in a *single family* had unprotected sex with whom and in what order. Pinch me it can't be true. Ouch! Shit, it is. The demise of monarchies in most countries offered a potential vacuum in which some form of free and fair society could arise and the Cult had that base covered. Monarchies had served its interests but they couldn't continue in the face of such widespread opposition and, anyway, replacing a 'royal' dictatorship that people could see with a dictatorship 'of the people' hiding behind the concept of 'democracy' presented far greater manipulative possibilities and ways of hiding coordinated tyranny behind the illusion of 'freedom'.

Democracy is quite wrongly defined as government selected by the population. This is not the case at all. It is government selected by *some* of the population (and then only in theory). This 'some' doesn't even have to be the majority as we have seen so often in first-past-the-post elections in which the so-called majority party wins fewer votes than the 'losing' parties combined. Democracy can give total power to a party in government from a minority of the votes cast. It's a sleight of hand to sell tyranny as freedom. Seventy-four million Trump-supporting Americans didn't vote for the 'Democratic' Party of Joe Biden in the distinctly dodgy election in 2020 and yet far from acknowledging the wishes and feelings of that great percentage of American society the Cult-owned Biden government set out from day one to destroy them and their right to a voice and opinion. Empty shell Biden and his Cult handlers said they were doing this to 'protect democracy'. Such is the level of lunacy and sickness to which politics has descended. Connect the dots and relate them to the desired outcome – a world government run by self-appointed technocrats and no longer even elected

politicians. While operating through its political agents in government the Cult is at the same time encouraging public distain for politicians by putting idiots and incompetents in theoretical power on the road to deleting them. The idea is to instil a public reaction that says of the technocrats: 'Well, they couldn't do any worse than the pathetic politicians.' It's all about controlling perception and Renegade Minds can see through that while programmed minds cannot when they are ignorant of both the planned outcome and the manipulation techniques employed to secure that end. This knowledge can be learned, however, and fast if people choose to get informed.

Politics may at first sight appear very difficult to control from a central point. I mean look at the 'different' parties and how would you be able to oversee them all and their constituent parts? In truth, it's very straightforward because of their structure. We are back to the pyramid of imposition and acquiescence. Organisations are structured in the same way as the system as a whole. Political parties are not open forums of free expression. They are hierarchies. I was a national spokesman for the British Green Party which claimed to be a different kind of politics in which influence and power was devolved; but I can tell you from direct experience – and it's far worse now – that Green parties are run as hierarchies like all the others however much they may try to hide that fact or kid themselves that it's not true. A very few at the top of all political parties are directing policy and personnel. They decide if you are elevated in the party or serve as a government minister and to do that you have to be a yes man or woman. Look at all the maverick political thinkers who never ascended the greasy pole. If you want to progress within the party or reach 'high-office' you need to fall into line and conform. Exceptions to this are rare indeed. Should you want to run for parliament or Congress you have to persuade the local or state level of the party to select you and for that you need to play the game as dictated by the hierarchy. If you secure election and wish to progress within the greater structure you need to go on conforming to what is acceptable to those running the hierarchy

from the peak of the pyramid. Political parties are perceptual gulags and the very fact that there are party 'Whips' appointed to 'whip' politicians into voting the way the hierarchy demands exposes the ridiculous idea that politicians are elected to serve the people they are supposed to represent. Cult operatives and manipulation has long seized control of major parties that have any chance of forming a government and at least most of those that haven't. A new party forms and the Cult goes to work to infiltrate and direct. This has reached such a level today that you see video compilations of 'leaders' of all parties whether Democrats, Republicans, Conservative, Labour and Green parroting the same Cult mantra of 'Build Back Better' and the 'Great Reset' which are straight off the Cult song-sheet to describe the transformation of global society in response to the Cult-instigated hoaxes of the 'Covid pandemic' and human-caused 'climate change'. To see Caroline Lucas, the Green Party MP that I knew when I was in the party in the 1980s, speaking in support of plans proposed by Cult operative Klaus Schwab representing the billionaire global elite is a real head-shaker.

Many parties – one master

The party system is another mind-trick and was instigated to change the nature of the dictatorship by swapping 'royalty' for dark suits that people believed – though now ever less so – represented their interests. Understanding this trick is to realise that a single force (the Cult) controls all parties either directly in terms of the major ones or through manipulation of perception and ideology with others. You don't need to manipulate Green parties to demand your transformation of society in the name of 'climate change' when they are obsessed with the lie that this is essential to 'save the planet'. You just give them a platform and away they go serving your interests while believing they are being environmentally virtuous. America's political structure is a perfect blueprint for how the two or multi-party system is really a one-party state. The Republican Party is controlled from one step back in the shadows by a group made up of billionaires and their gofers known as neoconservatives or Neocons.

I have exposed them in fine detail in my books and they were the driving force behind the policies of the imbecilic presidency of Boy George Bush which included 9/11 (see *The Trigger* for a comprehensive demolition of the official story), the subsequent 'war on terror' (war *of* terror) and the invasions of Afghanistan and Iraq. The latter was a No-Problem-Reaction-Solution based on claims by Cult operatives, including Bush and British Prime Minister Tony Blair, about Saddam Hussein's 'weapons of mass destruction' which did not exist as war criminals Bush and Blair well knew.

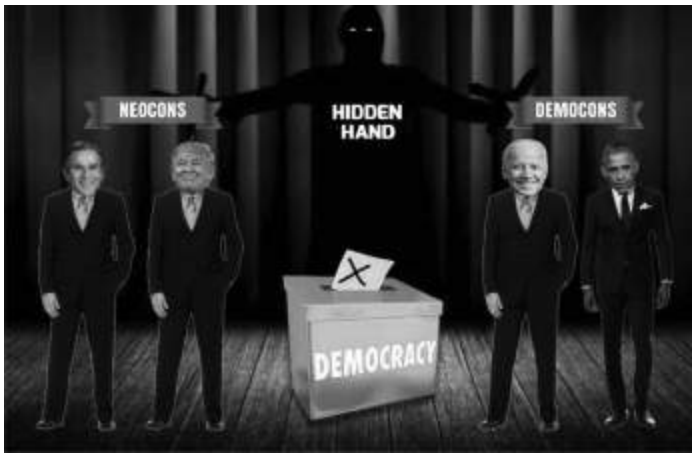


Figure 6: Different front people, different parties – same control system.

The Democratic Party has its own 'Neocon' group controlling from the background which I call the 'Democons' and here's the penny-drop – the Neocons and Democons answer to the same masters one step further back into the shadows (Fig 6). At that level of the Cult the Republican and Democrat parties are controlled by the same people and no matter which is in power the Cult is in power. This is how it works in almost every country and certainly in Britain with Conservative, Labour, Liberal Democrat and Green parties now all on the same page whatever the rhetoric may be in their feeble attempts to appear different. Neocons operated at the time of Bush through a think tank called The Project for the New American Century which in September, 2000, published a document entitled *Rebuilding America's Defenses: Strategies, Forces, and Resources*

For a New Century demanding that America fight 'multiple, simultaneous major theatre wars' as a 'core mission' to force regime-change in countries including Iraq, Libya and Syria. Neocons arranged for Bush ('Republican') and Blair ('Labour Party') to front-up the invasion of Iraq and when they departed the Democons orchestrated the targeting of Libya and Syria through Barack Obama ('Democrat') and British Prime Minister David Cameron ('Conservative Party'). We have 'different' parties and 'different' people, but the same unfolding script. The more the Cult has seized the reigns of parties and personnel the more their policies have transparently pursued the same agenda to the point where the fascist 'Covid' impositions of the Conservative junta of Jackboot Johnson in Britain were opposed by the Labour Party because they were not fascist enough. The Labour Party is likened to the US Democrats while the Conservative Party is akin to a British version of the Republicans and on both sides of the Atlantic they all speak the same language and support the direction demanded by the Cult although some more enthusiastically than others. It's a similar story in country after country because it's all centrally controlled. Oh, but what about Trump? I'll come to him shortly. Political 'choice' in the 'party' system goes like this: You vote for Party A and they get into government. You don't like what they do so next time you vote for Party B and they get into government. You don't like what they do when it's pretty much the same as Party A and why wouldn't that be with both controlled by the same force? Given that only two, sometimes three, parties have any chance of forming a government to get rid of Party B that you don't like you have to vote again for Party A which … you don't like. This, ladies and gentlemen, is what they call 'democracy' which we are told – wrongly – is a term interchangeable with 'freedom'.

The cult of cults

At this point I need to introduce a major expression of the Global Cult known as Sabbatian-Frankism. Sabbatian is also spelt as Sabbatean. I will summarise here. I have published major exposés

and detailed background in other works. Sabbatian-Frankism combines the names of two frauds posing as 'Jewish' men, Sabbatai Zevi (1626-1676), a rabbi, black magician and occultist who proclaimed he was the Jewish messiah; and Jacob Frank (1726-1791), the Polish 'Jew', black magician and occultist who said he was the reincarnation of 'messiah' Zevi and biblical patriarch Jacob. They worked across two centuries to establish the Sabbatian-Frankist cult that plays a major, indeed central, role in the manipulation of human society by the Global Cult which has its origins much further back in history than Sabbatai Zevi. I should emphasise two points here in response to the shrill voices that will scream 'anti-Semitism': (1) Sabbatian-Frankists are NOT Jewish and only pose as such to hide their cult behind a Jewish façade; and (2) my information about this cult has come from Jewish sources who have long realised that their society and community has been infiltrated and taken over by interloper Sabbatian-Frankists. Infiltration has been the foundation technique of Sabbatian-Frankism from its official origin in the 17th century. Zevi's Sabbatian sect attracted a massive following described as the biggest messianic movement in Jewish history, spreading as far as Africa and Asia, and he promised a return for the Jews to the 'Promised Land' of Israel. Sabbatianism was not Judaism but an inversion of everything that mainstream Judaism stood for. So much so that this sinister cult would have a feast day when Judaism had a fast day and whatever was forbidden in Judaism the Sabbatians were encouraged and even commanded to do. This included incest and what would be today called Satanism. Members were forbidden to marry outside the sect and there was a system of keeping their children ignorant of what they were part of until they were old enough to be trusted not to unknowingly reveal anything to outsiders. The same system is employed to this day by the Global Cult in general which Sabbatian-Frankism has enormously influenced and now largely controls.

Zevi and his Sabbatians suffered a setback with the intervention by the Sultan of the Islamic Ottoman Empire in the Middle East and what is now the Republic of Turkey where Zevi was located. The

Sultan gave him the choice of proving his 'divinity', converting to Islam or facing torture and death. Funnily enough Zevi chose to convert or at least appear to. Some of his supporters were disillusioned and drifted away, but many did not with 300 families also converting – only in theory – to Islam. They continued behind this Islamic smokescreen to follow the goals, rules and rituals of Sabbatianism and became known as 'crypto-Jews' or the 'Dönmeh' which means 'to turn'. This is rather ironic because they didn't 'turn' and instead hid behind a fake Islamic persona. The process of appearing to be one thing while being very much another would become the calling card of Sabbatianism especially after Zevi's death and the arrival of the Satanist Jacob Frank in the 18th century when the cult became Sabbatian-Frankism and plumbed still new depths of depravity and infiltration which included – still includes – human sacrifice and sex with children. Wherever Sabbatians go paedophilia and Satanism follow and is it really a surprise that Hollywood is so infested with child abuse and Satanism when it was established by Sabbatian-Frankists and is still controlled by them? Hollywood has been one of the prime vehicles for global perceptual programming and manipulation. How many believe the version of 'history' portrayed in movies when it is a travesty and inversion (again) of the truth? Rabbi Marvin Antelman describes Frankism in his book, *To Eliminate the Opiate*, as 'a movement of complete evil' while Jewish professor Gershom Scholem said of Frank in *The Messianic Idea in Judaism*: 'In all his actions [he was] a truly corrupt and degenerate individual ... one of the most frightening phenomena in the whole of Jewish history.' Frank was excommunicated by traditional rabbis, as was Zevi, but Frank was undeterred and enjoyed vital support from the House of Rothschild, the infamous banking dynasty whose inner-core are Sabbatian-Frankists and not Jews. Infiltration of the Roman Church and Vatican was instigated by Frank with many Dönmeh 'turning' again to convert to Roman Catholicism with a view to hijacking the reins of power. This was the ever-repeating modus operandi and continues to be so. Pose as an advocate of the religion, culture or country that you want to control and then

manipulate your people into the positions of authority and influence largely as advisers, administrators and Svengalis for those that appear to be in power. They did this with Judaism, Christianity (Christian Zionism is part of this), Islam and other religions and nations until Sabbatian-Frankism spanned the world as it does today.

Sabbatian Saudis and the terror network

One expression of the Sabbatian-Frankist Dönmeh within Islam is the ruling family of Saudi Arabia, the House of Saud, through which came the vile distortion of Islam known as Wahhabism. This is the violent creed followed by terrorist groups like Al-Qaeda and ISIS or Islamic State. Wahhabism is the hand-chopping, head-chopping 'religion' of Saudi Arabia which is used to keep the people in a constant state of fear so the interloper House of Saud can continue to rule. Al-Qaeda and Islamic State were lavishly funded by the House of Saud while being created and directed by the Sabbatian-Frankist network in the United States that operates through the Pentagon, CIA and the government in general of whichever 'party'. The front man for the establishment of Wahhabism in the middle of the 18th century was a Sabbatian-Frankist 'crypto-Jew' posing as Islamic called Muhammad ibn Abd al-Wahhab. His daughter would marry the son of Muhammad bin Saud who established the first Saudi state before his death in 1765 with support from the British Empire. Bin Saud's successors would establish modern Saudi Arabia in league with the British and Americans in 1932 which allowed them to seize control of Islam's major shrines in Mecca and Medina. They have dictated the direction of Sunni Islam ever since while Iran is the major centre of the Shiite version and here we have the source of at least the public conflict between them. The Sabbatian network has used its Wahhabi extremists to carry out Problem-Reaction-Solution terrorist attacks in the name of 'Al-Qaeda' and 'Islamic State' to justify a devastating 'war on terror', ever-increasing surveillance of the population and to terrify people into compliance. Another insight of the Renegade Mind is the streetwise understanding that

just because a country, location or people are attacked doesn't mean that those apparently representing that country, location or people are not behind the attackers. Often they are *orchestrating* the attacks because of the societal changes that can be then justified in the name of 'saving the population from terrorists'.

I show in great detail in *The Trigger* how Sabbatian-Frankists were the real perpetrators of 9/11 and not '19 Arab hijackers' who were blamed for what happened. Observe what was justified in the name of 9/11 alone in terms of Middle East invasions, mass surveillance and control that fulfilled the demands of the Project for the New American Century document published by the Sabbatian Neocons. What appear to be enemies are on the deep inside players on the same Sabbatian team. Israel and Arab 'royal' dictatorships are all ruled by Sabbatians and the recent peace agreements between Israel and Saudi Arabia, the United Arab Emirates (UAE) and others are only making formal what has always been the case behind the scenes. Palestinians who have been subjected to grotesque tyranny since Israel was bombed and terrorised into existence in 1948 have never stood a chance. Sabbatian-Frankists have controlled Israel (so the constant theme of violence and war which Sabbatians love) and they have controlled the Arab countries that Palestinians have looked to for real support that never comes. 'Royal families' of the Arab world in Saudi Arabia, Bahrain, UAE, etc., are all Sabbatians with allegiance to the aims of the cult and not what is best for their Arabic populations. They have stolen the oil and financial resources from their people by false claims to be 'royal dynasties' with a genetic right to rule and by employing vicious militaries to impose their will.

Satanic 'illumination'

The Satanist Jacob Frank formed an alliance in 1773 with two other Sabbatians, Mayer Amschel Rothschild (1744-1812), founder of the Rothschild banking dynasty, and Jesuit-educated fraudulent Jew, Adam Weishaupt, and this led to the formation of the Bavarian Illuminati, firstly under another name, in 1776. The Illuminati would

be the manipulating force behind the French Revolution (1789-1799) and was also involved in the American Revolution (1775-1783) before and after the Illuminati's official creation. Weishaupt would later become (in public) a Protestant Christian in archetypal Sabbatian style. I read that his name can be decoded as Adam-Weishaupt or 'the first man to lead those who know'. He wasn't a leader in the sense that he was a subordinate, but he did lead those below him in a crusade of transforming human society that still continues today. The theme was confirmed as early as 1785 when a horseman courier called Lanz was reported to be struck by lighting and extensive Illuminati documents were found in his saddlebags. They made the link to Weishaupt and detailed the plan for world takeover. Current events with 'Covid' fascism have been in the making for a very long time. Jacob Frank was jailed for 13 years by the Catholic Inquisition after his arrest in 1760 and on his release he headed for Frankfurt, Germany, home city and headquarters of the House of Rothschild where the alliance was struck with Mayer Amschel Rothschild and Weishaupt. Rothschild arranged for Frank to be given the title of Baron and he became a wealthy nobleman with a big following of Jews in Germany, the Austro-Hungarian Empire and other European countries. Most of them would have believed he was on their side.

The name 'Illuminati' came from the Zohar which is a body of works in the Jewish mystical 'bible' called the Kabbalah. 'Zohar' is the foundation of Sabbatian-Frankist belief and in Hebrew 'Zohar' means 'splendour', 'radiance', 'illuminated', and so we have 'Illuminati'. They claim to be the 'Illuminated Ones' from their knowledge systematically hidden from the human population and passed on through generations of carefully-chosen initiates in the global secret society network or Cult. Hidden knowledge includes an awareness of the Cult agenda for the world and the nature of our collective reality that I will explore later. Cult 'illumination' is symbolised by the torch held by the Statue of Liberty which was gifted to New York by French Freemasons in Paris who knew exactly what it represents. 'Liberty' symbolises the goddess worshipped in

Babylon as Queen Semiramis or Ishtar. The significance of this will become clear. Notice again the ubiquitous theme of inversion with the Statue of 'Liberty' really symbolising mass control (Fig 7). A mirror-image statute stands on an island in the River Seine in Paris from where New York Liberty originated (Fig 8). A large replica of the Liberty flame stands on top of the Pont de l'Alma tunnel in Paris where Princess Diana died in a Cult ritual described in *The Biggest Secret*. Lucifer 'the light bringer' is related to all this (and much more as we'll see) and 'Lucifer' is a central figure in Sabbatian-Frankism and its associated Satanism. Sabbatians reject the Jewish Torah, or Pentateuch, the 'five books of Moses' in the Old Testament known as Genesis, Exodus, Leviticus, Numbers, and Deuteronomy which are claimed by Judaism and Christianity to have been dictated by 'God' to Moses on Mount Sinai. Sabbatians say these do not apply to them and they seek to replace them with the Zohar to absorb Judaism and its followers into their inversion which is an expression of a much greater global inversion. They want to delete all religions and force humanity to worship a one-world religion – Sabbatian Satanism that also includes worship of the Earth goddess. Satanic themes are being more and more introduced into mainstream society and while Christianity is currently the foremost target for destruction the others are planned to follow.

Figure 7: The Cult goddess of Babylon disguised as the Statue of Liberty holding the flame of Lucifer the 'light bringer'.

Figure 8: Liberty's mirror image in Paris where the New York version originated.

Marx brothers

Rabbi Marvin Antelman connects the Illuminati to the Jacobins in *To Eliminate the Opiate* and Jacobins were the force behind the French Revolution. He links both to the Bund der Gerechten, or League of the Just, which was the network that inflicted communism/Marxism on the world. Antelman wrote:

> The original inner circle of the Bund der Gerechten consisted of born Catholics, Protestants and Jews [Sabbatian-Frankist infiltrators], and those representatives of respective subdivisions formulated schemes for the ultimate destruction of their faiths. The heretical Catholics laid plans which they felt would take a century or more for the ultimate destruction of the church; the apostate Jews for the ultimate destruction of the Jewish religion.

Sabbatian-created communism connects into this anti-religion agenda in that communism does not allow for the free practice of religion. The Sabbatian 'Bund' became the International Communist Party and Communist League and in 1848 'Marxism' was born with the Communist Manifesto of Sabbatian assets Karl Marx and Friedrich Engels. It is absolutely no coincidence that Marxism, just a different name for fascist and other centrally-controlled tyrannies, is being imposed worldwide as a result of the 'Covid' hoax and nor that Marxist/fascist China was the place where the hoax originated. The reason for this will become very clear in the chapter 'Covid: The calculated catastrophe'. The so-called 'Woke' mentality has hijacked

traditional beliefs of the political left and replaced them with far-right make-believe 'social justice' better known as Marxism. Woke will, however, be swallowed by its own perceived 'revolution' which is really the work of billionaires and billionaire corporations feigning being 'Woke'. Marxism is being touted by Wokers as a replacement for 'capitalism' when we don't have 'capitalism'. We have cartelism in which the market is stitched up by the very Cult billionaires and corporations bankrolling Woke. Billionaires love Marxism which keeps the people in servitude while they control from the top. Terminally naïve Wokers think they are 'changing the world' when it's the Cult that is doing the changing and when they have played their vital part and become surplus to requirements they, too, will be targeted. The Illuminati-Jacobins were behind the period known as 'The Terror' in the French Revolution in 1793 and 1794 when Jacobin Maximillian de Robespierre and his Orwellian 'Committee of Public Safety' killed 17,000 'enemies of the Revolution' who had once been 'friends of the Revolution'. Karl Marx (1818-1883), whose Sabbatian creed of Marxism has cost the lives of at least 100 million people, is a hero once again to Wokers who have been systematically kept ignorant of real history by their 'education' programming. As a result they now promote a Sabbatian 'Marxist' abomination destined at some point to consume them. Rabbi Antelman, who spent decades researching the Sabbatian plot, said of the League of the Just and Karl Marx:

> Contrary to popular opinion Karl Marx did not originate the Communist Manifesto. He was paid for his services by the League of the Just, which was known in its country of origin, Germany, as the Bund der Geaechteten.

Antelman said the text attributed to Marx was the work of other people and Marx 'was only repeating what others already said'. Marx was 'a hired hack – lackey of the wealthy Illuminists'. Marx famously said that religion was the 'opium of the people' (part of the Sabbatian plan to demonise religion) and Antelman called his books, *To Eliminate the Opiate*. Marx was born Jewish, but his family converted to Christianity (Sabbatian modus operandi) and he

attacked Jews, not least in his book, *A World Without Jews*. In doing so he supported the Sabbatian plan to destroy traditional Jewishness and Judaism which we are clearly seeing today with the vindictive targeting of orthodox Jews by the Sabbatian government of Israel over 'Covid' laws. I don't follow any religion and it has done much damage to the world over centuries and acted as a perceptual straightjacket. Renegade Minds, however, are always asking *why* something is being done. It doesn't matter if they agree or disagree with what is happening – *why* is it happening is the question. The 'why?' can be answered with regard to religion in that religions create interacting communities of believers when the Cult wants to dismantle all discourse, unity and interaction (see 'Covid' lockdowns) and the ultimate goal is to delete all religions for a one-world religion of Cult Satanism worshipping their 'god' of which more later. We see the same 'why?' with gun control in America. I don't have guns and don't want them, but why is the Cult seeking to disarm the population at the same time that law enforcement agencies are armed to their molars and why has every tyrant in history sought to disarm people before launching the final takeover? They include Hitler, Stalin, Pol Pot and Mao who followed confiscation with violent seizing of power. You know it's a Cult agenda by the people who immediately race to the microphones to exploit dead people in multiple shootings. Ultra-Zionist Cult lackey Senator Chuck Schumer was straight on the case after ten people were killed in Boulder, Colorado in March, 2121. Simple rule … if Schumer wants it the Cult wants it and the same with his ultra-Zionist mate the wild-eyed Senator Adam Schiff. At the same time they were calling for the disarmament of Americans, many of whom live a long way from a police response, Schumer, Schiff and the rest of these pampered clowns were sitting on Capitol Hill behind a razor-wired security fence protected by thousands of armed troops in addition to their own armed bodyguards. Mom and pop in an isolated home? They're just potential mass shooters.

Zion Mainframe

Sabbatian-Frankists and most importantly the Rothschilds were behind the creation of 'Zionism', a political movement that demanded a Jewish homeland in Israel as promised by Sabbatai Zevi. The very symbol of Israel comes from the German meaning of the name Rothschild. Dynasty founder Mayer Amschel Rothschild changed the family name from Bauer to Rothschild, or 'Red-Shield' in German, in deference to the six-pointed 'Star of David' hexagram displayed on the family's home in Frankfurt. The symbol later appeared on the flag of Israel after the Rothschilds were centrally involved in its creation. Hexagrams are not a uniquely Jewish symbol and are widely used in occult ('hidden') networks often as a symbol for Saturn (see my other books for why). Neither are Zionism and Jewishness interchangeable. Zionism is a political movement and philosophy and not a 'race' or a people. Many Jews oppose Zionism and many non-Jews, including US President Joe Biden, call themselves Zionists as does Israel-centric Donald Trump. America's support for the Israel government is pretty much a gimme with ultra-Zionist billionaires and corporations providing fantastic and dominant funding for both political parties. Former Congresswoman Cynthia McKinney has told how she was approached immediately she ran for office to 'sign the pledge' to Israel and confirm that she would always vote in that country's best interests. All American politicians are approached in this way. Anyone who refuses will get no support or funding from the enormous and all-powerful Zionist lobby that includes organisations like mega-lobby group AIPAC, the American Israel Public Affairs Committee. Trump's biggest funder was ultra-Zionist casino and media billionaire Sheldon Adelson while major funders of the Democratic Party include ultra-Zionist George Soros and ultra-Zionist financial and media mogul, Haim Saban. Some may reel back at the suggestion that Soros is an Israel-firster (Sabbatian-controlled Israel-firster), but Renegade Minds watch the actions not the words and everywhere Soros donates his billions the Sabbatian agenda benefits. In the spirit of Sabbatian inversion Soros pledged $1 billion for a new university network to promote 'liberal values and tackle intolerance'. He made the announcement during his annual speech

at the Cult-owned World Economic Forum in Davos, Switzerland, in January, 2020, after his 'harsh criticism' of 'authoritarian rulers' around the world. You can only laugh at such brazen mendacity. How *he* doesn't laugh is the mystery. Translated from the Orwellian 'liberal values and tackle intolerance' means teaching non-white people to hate white people and for white people to loathe themselves for being born white. The reason for that will become clear.

The 'Anti-Semitism' fraud

Zionists support the Jewish homeland in the land of Palestine which has been the Sabbatian-Rothschild goal for so long, but not for the benefit of Jews. Sabbatians and their global Anti-Semitism Industry have skewed public and political opinion to equate opposing the violent extremes of Zionism to be a blanket attack and condemnation of all Jewish people. Sabbatians and their global Anti-Semitism Industry have skewed public and political opinion to equate opposing the violent extremes of Zionism to be a blanket attack and condemnation of all Jewish people. This is nothing more than a Sabbatian protection racket to stop legitimate investigation and exposure of their agendas and activities. The official definition of 'anti-Semitism' has more recently been expanded to include criticism of Zionism – a *political movement* – and this was done to further stop exposure of Sabbatian infiltrators who created Zionism as we know it today in the 19th century. Renegade Minds will talk about these subjects when they know the shit that will come their way. People must decide if they want to know the truth or just cower in the corner in fear of what others will say. Sabbatians have been trying to label me as 'anti-Semitic' since the 1990s as I have uncovered more and more about their background and agendas. Useless, gutless, fraudulent 'journalists' then just repeat the smears without question and on the day I was writing this section a pair of unquestioning repeaters called Ben Quinn and Archie Bland (how appropriate) outright called me an 'anti-Semite' in the establishment propaganda sheet, the London *Guardian*, with no supporting evidence. The

Sabbatian Anti-Semitism Industry said so and who are they to question that? They wouldn't dare. Ironically 'Semitic' refers to a group of languages in the Middle East that are almost entirely Arabic. 'Anti-Semitism' becomes 'anti-Arab' which if the consequences of this misunderstanding were not so grave would be hilarious. Don't bother telling Quinn and Bland. I don't want to confuse them, bless 'em. One reason I am dubbed 'anti-Semitic' is that I wrote in the 1990s that Jewish operatives (Sabbatians) were heavily involved in the Russian Revolution when Sabbatians overthrew the Romanov dynasty. This apparently made me 'anti-Semitic'. Oh, really? Here is a section from *The Trigger*:

British journalist Robert Wilton confirmed these themes in his 1920 book *The Last Days of the Romanovs* when he studied official documents from the Russian government to identify the members of the Bolshevik ruling elite between 1917 and 1919. The Central Committee included 41 Jews among 62 members; the Council of the People's Commissars had 17 Jews out of 22 members; and 458 of the 556 most important Bolshevik positions between 1918 and 1919 were occupied by Jewish people. Only 17 were Russian. Then there were the 23 Jews among the 36 members of the vicious Cheka Soviet secret police established in 1917 who would soon appear all across the country.

Professor Robert Service of Oxford University, an expert on 20th century Russian history, found evidence that ['Jewish'] Leon Trotsky had sought to make sure that Jews were enrolled in the Red Army and were disproportionately represented in the Soviet civil bureaucracy that included the Cheka which performed mass arrests, imprisonment and executions of 'enemies of the people'. A US State Department Decimal File (861.00/5339) dated November 13th, 1918, names [Rothschild banking agent in America] Jacob Schiff and a list of ultra-Zionists as funders of the Russian Revolution leading to claims of a 'Jewish plot', but the key point missed by all is they were not 'Jews' – they were Sabbatian-Frankists.

Britain's Winston Churchill made the same error by mistake or otherwise. He wrote in a 1920 edition of the *Illustrated Sunday Herald* that those behind the Russian revolution were part of a 'worldwide conspiracy for the overthrow of civilisation and for the reconstitution of society on the basis of arrested development, of envious malevolence, and impossible equality' (see 'Woke' today because that has been created by the same network). Churchill said there was no need to exaggerate the part played in the creation of Bolshevism and in the actual bringing about of the Russian

Revolution 'by these international and for the most part atheistical Jews' ['atheistical Jews' = Sabbatians]. Churchill said it is certainly a very great one and probably outweighs all others: 'With the notable exception of Lenin, the majority of the leading figures are Jews.' He went on to describe, knowingly or not, the Sabbatian modus operandi of placing puppet leaders nominally in power while they control from the background:

> Moreover, the principal inspiration and driving power comes from the Jewish leaders. Thus Tchitcherin, a pure Russian, is eclipsed by his nominal subordinate, Litvinoff, and the influence of Russians like Bukharin or Lunacharski cannot be compared with the power of Trotsky, or of Zinovieff, the Dictator of the Red Citadel (Petrograd), or of Krassin or Radek – all Jews. In the Soviet institutions the predominance of Jews is even more astonishing. And the prominent, if not indeed the principal, part in the system of terrorism applied by the Extraordinary Commissions for Combatting Counter-Revolution has been taken by Jews, and in some notable cases by Jewesses.

What I said about seriously disproportionate involvement in the Russian Revolution by Jewish 'revolutionaries' (Sabbatians) is provable fact, but truth is no defence against the Sabbatian Anti-Semitism Industry, its repeater parrots like Quinn and Bland, and the now breathtaking network of so-called 'Woke' 'anti-hate' groups with interlocking leaderships and funding which have the role of discrediting and silencing anyone who gets too close to exposing the Sabbatians. We have seen 'truth is no defence' confirmed in legal judgements with the Saskatchewan Human Rights Commission in Canada decreeing this: 'Truthful statements can be presented in a manner that would meet the definition of hate speech, and not all truthful statements must be free from restriction.' Most 'anti-hate' activists, who are themselves consumed by hatred, are too stupid and ignorant of the world to know how they are being used. They are far too far up their own virtue-signalling arses and it's far too dark for them to see anything.

The 'revolution' game

The background and methods of the 'Russian' Revolution are straight from the Sabbatian playbook seen in the French Revolution

and endless others around the world that appear to start as a revolution of the people against tyrannical rule and end up with a regime change to more tyrannical rule overtly or covertly. Wars, terror attacks and regime overthrows follow the Sabbatian cult through history with its agents creating them as Problem-Reaction-Solutions to remove opposition on the road to world domination. Sabbatian dots connect the Rothschilds with the Illuminati, Jacobins of the French Revolution, the 'Bund' or League of the Just, the International Communist Party, Communist League and the Communist Manifesto of Karl Marx and Friedrich Engels that would lead to the Rothschild-funded Russian Revolution. The sequence comes under the heading of 'creative destruction' when you advance to your global goal by continually destroying the status quo to install a new status quo which you then also destroy. The two world wars come to mind. With each new status quo you move closer to your planned outcome. Wars and mass murder are to Sabbatians a collective blood sacrifice ritual. They are obsessed with death for many reasons and one is that death is an inversion of life. Satanists and Sabbatians are obsessed with death and often target churches and churchyards for their rituals. Inversion-obsessed Sabbatians explain the use of inverted symbolism including the *inverted* pentagram and *inverted* cross. The inversion of the cross has been related to targeting Christianity, but the cross was a religious symbol long before Christianity and its inversion is a statement about the Sabbatian mentality and goals more than any single religion.

Sabbatians operating in Germany were behind the rise of the occult-obsessed Nazis and the subsequent Jewish exodus from Germany and Europe to Palestine and the United States after World War Two. The Rothschild dynasty was at the forefront of this both as political manipulators and by funding the operation. Why would Sabbatians help to orchestrate the horrors inflicted on Jews by the Nazis and by Stalin after they organised the Russian Revolution? Sabbatians hate Jews and their religion, that's why. They pose as Jews and secure positions of control within Jewish society and play the 'anti-Semitism' card to protect themselves from exposure

through a global network of organisations answering to the Sabbatian-created-and-controlled globe-spanning intelligence network that involves a stunning web of military-intelligence operatives and operations for a tiny country of just nine million. Among them are Jewish assets who are not Sabbatians but have been convinced by them that what they are doing is for the good of Israel and the Jewish community to protect them from what they have been programmed since childhood to believe is a Jew-hating hostile world. The Jewish community is just a highly convenient cover to hide the true nature of Sabbatians. Anyone getting close to exposing their game is accused by Sabbatian place-people and gofers of 'anti-Semitism' and claiming that all Jews are part of a plot to take over the world. I am not saying that. I am saying that Sabbatians – the *real* Jew-haters – have infiltrated the Jewish community to use them both as a cover and an 'anti-Semitic' defence against exposure. Thus we have the Anti-Semitism Industry targeted researchers in this way and most Jewish people think this is justified and genuine. They don't know that their 'Jewish' leaders and institutions of state, intelligence and military are not controlled by Jews at all, but cultists and stooges of Sabbatian-Frankism. I once added my name to a pro-Jewish freedom petition online and the next time I looked my name was gone and text had been added to the petition blurb to attack me as an 'anti-Semite' such is the scale of perceptual programming.

Moving on America

I tell the story in *The Trigger* and a chapter called 'Atlantic Crossing' how particularly after Israel was established the Sabbatians moved in on the United States and eventually grasped control of government administration, the political system via both Democrats and Republicans, the intelligence community like the CIA and National Security Agency (NSA), the Pentagon and mass media. Through this seriously compartmentalised network Sabbatians and their operatives in Mossad, Israeli Defense Forces (IDF) and US agencies pulled off 9/11 and blamed it on 19 'Al-Qaeda hijackers' dominated by men from, or connected to, Sabbatian-ruled Saudi

Arabia. The '19' were not even on the planes let alone flew those big passenger jets into buildings while being largely incompetent at piloting one-engine light aircraft. 'Hijacker' Hani Hanjour who is said to have flown American Airlines Flight 77 into the Pentagon with a turn and manoeuvre most professional pilots said they would have struggled to do was banned from renting a small plane by instructors at the Freeway Airport in Bowie, Maryland, just *six weeks* earlier on the grounds that he was an incompetent pilot. The Jewish population of the world is just 0.2 percent with even that almost entirely concentrated in Israel (75 percent Jewish) and the United States (around two percent). This two percent and globally 0.2 percent refers to *Jewish* people and not Sabbatian interlopers who are a fraction of that fraction. What a sobering thought when you think of the fantastic influence on world affairs of tiny Israel and that the Project for the New America Century (PNAC) which laid out the blueprint in September, 2000, for America's war on terror and regime change wars in Iraq, Libya and Syria was founded and dominated by Sabbatians known as 'Neocons'. The document conceded that this plan would not be supported politically or publicly without a major attack on American soil and a Problem-Reaction-Solution excuse to send troops to war across the Middle East. Sabbatian Neocons said:

> ... [The] process of transformation ... [war and regime change] ... is likely to be a long one, absent some catastrophic and catalysing event – like a new Pearl Harbor.

Four months later many of those who produced that document came to power with their inane puppet George Bush from the long-time Sabbatian Bush family. They included Sabbatian Dick Cheney who was officially vice-president, but really de-facto president for the entirety of the 'Bush' government. Nine months after the 'Bush' inauguration came what Bush called at the time 'the Pearl Harbor of the 21st century' and with typical Sabbatian timing and symbolism 2001 was the 60th anniversary of the attack in 1941 by the Japanese Air Force on Pearl Harbor, Hawaii, which allowed President Franklin Delano Roosevelt to take the United States into a Sabbatian-

instigated Second World War that he said in his election campaign that he never would. The evidence is overwhelming that Roosevelt and his military and intelligence networks knew the attack was coming and did nothing to stop it, but they did make sure that America's most essential naval ships were not in Hawaii at the time. Three thousand Americans died in the Pearl Harbor attacks as they did on September 11th. By the 9/11 year of 2001 Sabbatians had widely infiltrated the US government, military and intelligence operations and used their compartmentalised assets to pull off the 'Al-Qaeda' attacks. If you read *The Trigger* it will blow your mind to see the utterly staggering concentration of 'Jewish' operatives (Sabbatian infiltrators) in essential positions of political, security, legal, law enforcement, financial and business power before, during, and after the attacks to make them happen, carry them out, and then cover their tracks – and I do mean *staggering* when you think of that 0.2 percent of the world population and two percent of Americans which are Jewish while Sabbatian infiltrators are a fraction of that. A central foundation of the 9/11 conspiracy was the hijacking of government, military, Air Force and intelligence computer systems in real time through 'back-door' access made possible by Israeli (Sabbatian) 'cyber security' software. Sabbatian-controlled Israel is on the way to rivalling Silicon Valley for domination of cyberspace and is becoming the dominant force in cyber-security which gives them access to entire computer systems and their passcodes across the world. Then add to this that Zionists head (officially) Silicon Valley giants like Google (Larry Page and Sergey Brin), Google-owned YouTube (Susan Wojcicki), Facebook (Mark Zuckerberg and Sheryl Sandberg), and Apple (Chairman Arthur D. Levinson), and that ultra-Zionist hedge fund billionaire Paul Singer has a $1 billion stake in Twitter which is only nominally headed by 'CEO' pothead Jack Dorsey. As cable news host Tucker Carlson said of Dorsey: 'There used to be debate in the medical community whether dropping a ton of acid had permanent effects and I think that debate has now ended.' Carlson made the comment after Dorsey told a hearing on Capitol Hill (if you cut through his bullshit) that he

believed in free speech so long as he got to decide what you can hear and see. These 'big names' of Silicon Valley are only front men and women for the Global Cult, not least the Sabbatians, who are the true controllers of these corporations. Does anyone still wonder why these same people and companies have been ferociously censoring and banning people (like me) for exposing any aspect of the Cult agenda and especially the truth about the 'Covid' hoax which Sabbatians have orchestrated?

The Jeffrey Epstein paedophile ring was a Sabbatian operation. He was officially 'Jewish' but he was a Sabbatian and women abused by the ring have told me about the high number of 'Jewish' people involved. The Epstein horror has Sabbatian written all over it and matches perfectly their modus operandi and obsession with sex and ritual. Epstein was running a Sabbatian blackmail ring in which famous people with political and other influence were provided with young girls for sex while everything was being filmed and recorded on hidden cameras and microphones at his New York house, Caribbean island and other properties. Epstein survivors have described this surveillance system to me and some have gone public. Once the famous politician or other figure knew he or she was on video they tended to do whatever they were told. Here we go again …when you've got them by the balls their hearts and minds will follow. Sabbatians use this blackmail technique on a wide scale across the world to entrap politicians and others they need to act as demanded. Epstein's private plane, the infamous 'Lolita Express', had many well-known passengers including Bill Clinton while Bill Gates has flown on an Epstein plane and met with him four years after Epstein had been jailed for paedophilia. They subsequently met many times at Epstein's home in New York according to a witness who was there. Epstein's infamous side-kick was Ghislaine Maxwell, daughter of Mossad agent and ultra-Zionist mega-crooked British businessman, Bob Maxwell, who at one time owned the *Daily Mirror* newspaper. Maxwell was murdered at sea on his boat in 1991 by Sabbatian-controlled Mossad when he became a liability with his

business empire collapsing as a former Mossad operative has confirmed (see *The Trigger*).

Money, money, money, funny money …

Before I come to the Sabbatian connection with the last three US presidents I will lay out the crucial importance to Sabbatians of controlling banking and finance. Sabbatian Mayer Amschel Rothschild set out to dominate this arena in his family's quest for total global control. What is freedom? It is, in effect, choice. The more choices you have the freer you are and the fewer your choices the more you are enslaved. In the global structure created over centuries by Sabbatians the biggest decider and restrictor of choice is … money. Across the world if you ask people what they would like to do with their lives and why they are not doing that they will reply 'I don't have the money'. This is the idea. A global elite of multi-billionaires are described as 'greedy' and that is true on one level; but control of money – who has it and who doesn't – is not primarily about greed. It's about control. Sabbatians have seized ever more control of finance and sucked the wealth of the world out of the hands of the population. We talk now, after all, about the 'One-percent' and even then the wealthiest are a lot fewer even than that. This has been made possible by a money scam so outrageous and so vast it could rightly be called the scam of scams founded on creating 'money' out of nothing and 'loaning' that with interest to the population. Money out of nothing is called 'credit'. Sabbatians have asserted control over governments and banking ever more completely through the centuries and secured financial laws that allow banks to lend hugely more than they have on deposit in a confidence trick known as fractional reserve lending. Imagine if you could lend money that doesn't exist and charge the recipient interest for doing so. You would end up in jail. Bankers by contrast end up in mansions, private jets, Malibu and Monaco.

Banks are only required to keep a fraction of their deposits and wealth in their vaults and they are allowed to lend 'money' they don't have called 'credit. Go into a bank for a loan and if you succeed

the banker will not move any real wealth into your account. They will type into your account the amount of the agreed 'loan' – say £100,000. This is not wealth that really exists; it is non-existent, fresh-air, created-out-of-nothing 'credit' which has never, does not, and will never exist except in theory. Credit is backed by nothing except wind and only has buying power because people think that it has buying power and accept it in return for property, goods and services. I have described this situation as like those cartoon characters you see chasing each other and when they run over the edge of a cliff they keep running forward on fresh air until one of them looks down, realises what's happened, and they all crash into the ravine. The whole foundation of the Sabbatian financial system is to stop people looking down except for periodic moments when they want to crash the system (as in 2008 and 2020 ongoing) and reap the rewards from all the property, businesses and wealth their borrowers had signed over as 'collateral' in return for a 'loan' of fresh air. Most people think that money is somehow created by governments when it comes into existence from the start as a debt through banks 'lending' illusory money called credit. Yes, the very currency of exchange is a *debt* from day one issued as an interest-bearing loan. Why don't governments create money interest-free and lend it to their people interest-free? Governments are controlled by Sabbatians and the financial system is controlled by Sabbatians for whom interest-free money would be a nightmare come true. Sabbatians underpin their financial domination through their global network of central banks, including the privately-owned US Federal Reserve and Britain's Bank of England, and this is orchestrated by a privately-owned central bank coordination body called the Bank for International Settlements in Basle, Switzerland, created by the usual suspects including the Rockefellers and Rothschilds. Central bank chiefs don't answer to governments or the people. They answer to the Bank for International Settlements or, in other words, the Global Cult which is dominated today by Sabbatians.

Built-in disaster

There are so many constituent scams within the overall banking scam. When you take out a loan of thin-air credit only the amount of that loan is theoretically brought into circulation to add to the amount in circulation; but you are paying back the principle plus interest. The additional interest is not created and this means that with every 'loan' there is a shortfall in the money in circulation between what is borrowed and what has to be paid back. There is never even close to enough money in circulation to repay all outstanding public and private debt including interest. Coldly weaved in the very fabric of the system is the certainty that some will lose their homes, businesses and possessions to the banking 'lender'. This is less obvious in times of 'boom' when the amount of money in circulation (and the debt) is expanding through more people wanting and getting loans. When a downturn comes and the money supply contracts it becomes painfully obvious that there is not enough money to service all debt and interest. This is less obvious in times of 'boom' when the amount of money in circulation (and the debt) is expanding through more people wanting and getting loans. When a downturn comes and the money supply contracts and it becomes painfully obvious – as in 2008 and currently – that there is not enough money to service all debt and interest. Sabbatian banksters have been leading the human population through a calculated series of booms (more debt incurred) and busts (when the debt can't be repaid and the banks get the debtor's tangible wealth in exchange for non-existent 'credit'). With each 'bust' Sabbatian bankers have absorbed more of the world's tangible wealth and we end up with the One-percent. Governments are in bankruptcy levels of debt to the same system and are therefore owned by a system they do not control. The Federal Reserve, 'America's central bank', is privately-owned and American presidents only nominally appoint its chairman or woman to maintain the illusion that it's an arm of government. It's not. The 'Fed' is a cartel of private banks which handed billions to its associates and friends after the crash of 2008 and has been Sabbatian-controlled since it was manipulated into being in 1913 through the covert trickery of Rothschild banking agents Jacob Schiff and Paul

Warburg, and the Sabbatian Rockefeller family. Somehow from a Jewish population of two-percent and globally 0.2 percent (Sabbatian interlopers remember are far smaller) ultra-Zionists headed the Federal Reserve for 31 years between 1987 and 2018 in the form of Alan Greenspan, Bernard Bernanke and Janet Yellen (now Biden's Treasury Secretary) with Yellen's deputy chairman a Israeli-American duel citizen and ultra-Zionist Stanley Fischer, a former governor of the Bank of Israel. Ultra-Zionist Fed chiefs spanned the presidencies of Ronald Reagan ('Republican'), Father George Bush ('Republican'), Bill Clinton ('Democrat'), Boy George Bush ('Republican') and Barack Obama ('Democrat'). We should really add the pre-Greenspan chairman, Paul Adolph Volcker, 'appointed' by Jimmy Carter ('Democrat') who ran the Fed between 1979 and 1987 during the Carter and Reagan administrations before Greenspan took over. Volcker was a long-time associate and business partner of the Rothschilds. No matter what the 'party' officially in power the United States economy was directed by the same force. Here are members of the Obama, Trump and Biden administrations and see if you can make out a common theme.

Barack Obama ('Democrat')

Ultra-Zionists Robert Rubin, Larry Summers, and Timothy Geithner ran the US Treasury in the Clinton administration and two of them reappeared with Obama. Ultra-Zionist Fed chairman Alan Greenspan had manipulated the crash of 2008 through deregulation and jumped ship just before the disaster to make way for ultra-Zionist Bernard Bernanke to hand out trillions to Sabbatian 'too big to fail' banks and businesses, including the ubiquitous ultra-Zionist Goldman Sachs which has an ongoing staff revolving door operation between itself and major financial positions in government worldwide. Obama inherited the fallout of the crash when he took office in January, 2009, and fortunately he had the support of his ultra-Zionist White House Chief of Staff Rahm Emmanuel, son of a terrorist who helped to bomb Israel into being in 1948, and his ultra-Zionist senior adviser David Axelrod, chief strategist in Obama's two

successful presidential campaigns. Emmanuel, later mayor of Chicago and former senior fundraiser and strategist for Bill Clinton, is an example of the Sabbatian policy after Israel was established of migrating insider families to America so their children would be born American citizens. 'Obama' chose this financial team throughout his administration to respond to the Sabbatian-instigated crisis:

Timothy Geithner (ultra-Zionist) Treasury Secretary; Jacob J. Lew, Treasury Secretary; Larry Summers (ultra-Zionist), director of the White House National Economic Council; Paul Adolph Volcker (Rothschild business partner), chairman of the Economic Recovery Advisory Board; Peter Orszag (ultra-Zionist), director of the Office of Management and Budget overseeing all government spending; Penny Pritzker (ultra-Zionist), Commerce Secretary; Jared Bernstein (ultra-Zionist), chief economist and economic policy adviser to Vice President Joe Biden; Mary Schapiro (ultra-Zionist), chair of the Securities and Exchange Commission (SEC); Gary Gensler (ultra-Zionist), chairman of the Commodity Futures Trading Commission (CFTC); Sheila Bair (ultra-Zionist), chair of the Federal Deposit Insurance Corporation (FDIC); Karen Mills (ultra-Zionist), head of the Small Business Administration (SBA); Kenneth Feinberg (ultra-Zionist), Special Master for Executive [bail-out] Compensation. Feinberg would be appointed to oversee compensation (with strings) to 9/11 victims and families in a campaign to stop them having their day in court to question the official story. At the same time ultra-Zionist Bernard Bernanke was chairman of the Federal Reserve and these are only some of the ultra-Zionists with allegiance to Sabbatian-controlled Israel in the Obama government. Obama's biggest corporate donor was ultra-Zionist Goldman Sachs which had employed many in his administration.

Donald Trump ('Republican')

Trump claimed to be an outsider (he wasn't) who had come to 'drain the swamp'. He embarked on this goal by immediately appointing ultra-Zionist Steve Mnuchin, a Goldman Sachs employee for 17

years, as his Treasury Secretary. Others included Gary Cohn (ultra-Zionist), chief operating officer of Goldman Sachs, his first Director of the National Economic Council and chief economic adviser, who was later replaced by Larry Kudlow (ultra-Zionist). Trump's senior adviser throughout his four years in the White House was his sinister son-in-law Jared Kushner, a life-long friend of Israel Prime Minister Benjamin Netanyahu. Kushner is the son of a convicted crook who was pardoned by Trump in his last days in office. Other ultra-Zionists in the Trump administration included: Stephen Miller, Senior Policy Adviser; Avrahm Berkowitz, Deputy Adviser to Trump and his Senior Adviser Jared Kushner; Ivanka Trump, Adviser to the President, who converted to Judaism when she married Jared Kushner; David Friedman, Trump lawyer and Ambassador to Israel; Jason Greenblatt, Trump Organization executive vice president and chief legal officer, who was made Special Representative for International Negotiations and the Israeli-Palestinian Conflict; Rod Rosenstein, Deputy Attorney General; Elliot Abrams, Special Representative for Venezuela, then Iran; John Eisenberg, National Security Council Legal Adviser and Deputy Council to the President for National Security Affairs; Anne Neuberger, Deputy National Manager, National Security Agency; Ezra Cohen-Watnick, Acting Under Secretary of Defense for Intelligence; Elan Carr, Special Envoy to monitor and combat anti-Semitism; Len Khodorkovsky, Deputy Special Envoy to monitor and combat anti-Semitism; Reed Cordish, Assistant to the President, Intragovernmental and Technology Initiatives. Trump Vice President Mike Pence and Secretary of State Mike Pompeo, both Christian Zionists, were also vehement supporters of Israel and its goals and ambitions.

Donald 'free-speech believer' Trump pardoned a number of financial and violent criminals while ignoring calls to pardon Julian Assange and Edward Snowden whose crimes are revealing highly relevant information about government manipulation and corruption and the widespread illegal surveillance of the American people by US 'security' agencies. It's so good to know that Trump is on the side of freedom and justice and not mega-criminals with

allegiance to Sabbatian-controlled Israel. These included a pardon for Israeli spy Jonathan Pollard who was jailed for life in 1987 under the Espionage Act. Aviem Sella, the Mossad agent who recruited Pollard, was also pardoned by Trump while Assange sat in jail and Snowden remained in exile in Russia. Sella had 'fled' (was helped to escape) to Israel in 1987 and was never extradited despite being charged under the Espionage Act. A Trump White House statement said that Sella's clemency had been 'supported by Benjamin Netanyahu, Ron Dermer, Israel's US Ambassador, David Friedman, US Ambassador to Israel and Miriam Adelson, wife of leading Trump donor Sheldon Adelson who died shortly before. Other friends of Jared Kushner were pardoned along with Sholom Weiss who was believed to be serving the longest-ever white-collar prison sentence of more than 800 years in 2000. The sentence was commuted of Ponzi-schemer Eliyahu Weinstein who defrauded Jews and others out of $200 million. I did mention that Assange and Snowden were ignored, right? Trump gave Sabbatians almost everything they asked for in military and political support, moving the US Embassy from Tel Aviv to Jerusalem with its critical symbolic and literal implications for Palestinian statehood, and the 'deal of the Century' designed by Jared Kushner and David Friedman which gave the Sabbatian Israeli government the green light to substantially expand its already widespread program of building illegal Jewish-only settlements in the occupied land of the West Bank. This made a two-state 'solution' impossible by seizing all the land of a potential Palestinian homeland and that had been the plan since 1948 and then 1967 when the Arab-controlled Gaza Strip, West Bank, Sinai Peninsula and Syrian Golan Heights were occupied by Israel. All the talks about talks and road maps and delays have been buying time until the West Bank was physically occupied by Israeli real estate. Trump would have to be a monumentally ill-informed idiot not to see that this was the plan he was helping to complete. The Trump administration was in so many ways the Kushner administration which means the Netanyahu administration which means the Sabbatian administration. I understand why many opposing Cult fascism in all its forms gravitated to Trump, but he

was a crucial part of the Sabbatian plan and I will deal with this in the next chapter.

Joe Biden ('Democrat')

A barely cognitive Joe Biden took over the presidency in January, 2021, along with his fellow empty shell, Vice-President Kamala Harris, as the latest Sabbatian gofers to enter the White House. Names on the door may have changed and the 'party' – the force behind them remained the same as Zionists were appointed to a stream of pivotal areas relating to Sabbatian plans and policy. They included: Janet Yellen, Treasury Secretary, former head of the Federal Reserve, and still another ultra-Zionist running the US Treasury after Mnuchin (Trump), Lew and Geithner (Obama), and Summers and Rubin (Clinton); Anthony Blinken, Secretary of State; Wendy Sherman, Deputy Secretary of State (so that's 'Biden's' Sabbatian foreign policy sorted); Jeff Zients, White House coronavirus coordinator; Rochelle Walensky, head of the Centers for Disease Control; Rachel Levine, transgender deputy health secretary (that's 'Covid' hoax policy under control); Merrick Garland, Attorney General; Alejandro Mayorkas, Secretary of Homeland Security; Cass Sunstein, Homeland Security with responsibility for new immigration laws; Avril Haines, Director of National Intelligence; Anne Neuberger, National Security Agency cybersecurity director (note, cybersecurity); David Cohen, CIA Deputy Director; Ronald Klain, Biden's Chief of Staff (see Rahm Emanuel); Eric Lander, a 'leading geneticist', Office of Science and Technology Policy director (see Smart Grid, synthetic biology agenda); Jessica Rosenworcel, acting head of the Federal Communications Commission (FCC) which controls Smart Grid technology policy and electromagnetic communication systems including 5G. How can it be that so many pivotal positions are held by two-percent of the American population and 0.2 percent of the world population administration after administration no matter who is the president and what is the party? It's a coincidence? Of course it's not and this is why Sabbatians have built their colossal global web of interlocking 'anti-

hate' hate groups to condemn anyone who asks these glaring questions as an 'anti-Semite'. The way that Jewish people horrifically abused in Sabbatian-backed Nazi Germany are exploited to this end is stomach-turning and disgusting beyond words.

Political fusion

Sabbatian manipulation has reversed the roles of Republicans and Democrats and the same has happened in Britain with the Conservative and Labour Parties. Republicans and Conservatives were always labelled the 'right' and Democrats and Labour the 'left', but look at the policy positions now and the Democrat-Labour 'left' has moved further to the 'right' than Republicans and Conservatives under the banner of 'Woke', the Cult-created far-right tyranny. Where once the Democrat-Labour 'left' defended free speech and human rights they now seek to delete them and as I said earlier despite the 'Covid' fascism of the Jackboot Johnson Conservative government in the UK the Labour Party of leader Keir Starmer demanded even more extreme measures. The Labour Party has been very publicly absorbed by Sabbatians after a political and media onslaught against the previous leader, the weak and inept Jeremy Corbyn, over made-up allegations of 'anti-Semitism' both by him and his party. The plan was clear with this 'anti-Semite' propaganda and what was required in response was a swift and decisive 'fuck off' from Corbyn and a statement to expose the Anti-Semitism Industry (Sabbatian) attempt to silence Labour criticism of the Israeli government (Sabbatians) and purge the party of all dissent against the extremes of ultra-Zionism (Sabbatians). Instead Corbyn and his party fell to their knees and appeased the abusers which, by definition, is impossible. Appeasing one demand leads only to a new demand to be appeased until takeover is complete. Like I say – 'fuck off' would have been a much more effective policy and I have used it myself with great effect over the years when Sabbatians are on my case which is most of the time. I consider that fact a great compliment, by the way. The outcome of the Labour Party capitulation is that we now have a Sabbatian-controlled

Conservative Party 'opposed' by a Sabbatian-controlled Labour Party in a one-party Sabbatian state that hurtles towards the extremes of tyranny (the Sabbatian cult agenda). In America the situation is the same. Labour's Keir Starmer spends his days on his knees with his tongue out pointing to Tel Aviv, or I guess now Jerusalem, while Boris Johnson has an 'anti-Semitism czar' in the form of former Labour MP John Mann who keeps Starmer company on his prayer mat.

Sabbatian influence can be seen in Jewish members of the Labour Party who have been ejected for criticism of Israel including those from families that suffered in Nazi Germany. Sabbatians despise real Jewish people and target them even more harshly because it is so much more difficult to dub them 'anti-Semitic' although in their desperation they do try.

CHAPTER THREE

The Pushbacker sting

Until you realize how easy it is for your mind to be manipulated, you remain the puppet of someone else's game
Evita Ochel

I will use the presidencies of Trump and Biden to show how the manipulation of the one-party state plays out behind the illusion of political choice across the world. No two presidencies could – on the face of it – be more different and apparently at odds in terms of direction and policy.

A Renegade Mind sees beyond the obvious and focuses on outcomes and consequences and not image, words and waffle. The Cult embarked on a campaign to divide America between those who blindly support its agenda (the mentality known as 'Woke') and those who are pushing back on where the Cult and its Sabbatians want to go. This presents infinite possibilities for dividing and ruling the population by setting them at war with each other and allows a perceptual ring fence of demonisation to encircle the Pushbackers in a modern version of the Little Big Horn in 1876 when American cavalry led by Lieutenant Colonel George Custer were drawn into a trap, surrounded and killed by Native American tribes defending their land of thousands of years from being seized by the government. In this modern version the roles are reversed and it's those defending themselves from the Sabbatian government who are surrounded and the government that's seeking to destroy them. This trap was set years ago and to explain how we must return to 2016

and the emergence of Donald Trump as a candidate to be President of the United States. He set out to overcome the best part of 20 other candidates in the Republican Party before and during the primaries and was not considered by many in those early stages to have a prayer of living in the White House. The Republican Party was said to have great reservations about Trump and yet somehow he won the nomination. When you know how American politics works – politics in general – there is no way that Trump could have become the party's candidate unless the Sabbatian-controlled 'Neocons' that run the Republican Party wanted that to happen. We saw the proof in emails and documents made public by WikiLeaks that the Democratic Party hierarchy, or Democons, systematically undermined the campaign of Bernie Sanders to make sure that Sabbatian gofer Hillary Clinton won the nomination to be their presidential candidate. If the Democons could do that then the Neocons in the Republican Party could have derailed Trump in the same way. But they didn't and at that stage I began to conclude that Trump could well be the one chosen to be president. If that was the case the 'why' was pretty clear to see – the goal of dividing America between Cult agenda-supporting Wokers and Pushbackers who gravitated to Trump because he was telling them what they wanted to hear. His constituency of support had been increasingly ignored and voiceless for decades and profoundly through the eight years of Sabbatian puppet Barack Obama. Now here was someone speaking their language of pulling back from the incessant globalisation of political and economic power, the exporting of American jobs to China and elsewhere by 'American' (Sabbatian) corporations, the deletion of free speech, and the mass immigration policies that had further devastated job opportunities for the urban working class of all races and the once American heartlands of the Midwest.

Beware the forked tongue

Those people collectively sighed with relief that at last a political leader was apparently on their side, but another trait of the Renegade Mind is that you look even harder at people telling you

what you want to hear than those who are telling you otherwise. Obviously as I said earlier people wish what they want to hear to be true and genuine and they are much more likely to believe that than someone saying what they don't want to here and don't want to be true. Sales people are taught to be skilled in eliciting by calculated questioning what their customers want to hear and repeating that back to them as their own opinion to get their targets to like and trust them. Assets of the Cult are also sales people in the sense of selling perception. To read Cult manipulation you have to play the long and expanded game and not fall for the Vaudeville show of party politics. Both American parties are vehicles for the Cult and they exploit them in different ways depending on what the agenda requires at that moment. Trump and the Republicans were used to be the focus of dividing America and isolating Pushbackers to open the way for a Biden presidency to become the most extreme in American history by advancing the full-blown Woke (Cult) agenda with the aim of destroying and silencing Pushbackers now labelled Nazi Trump supporters and white supremacists.

Sabbatians wanted Trump in office for the reasons described by ultra-Zionist Saul Alinsky (1909-1972) who was promoting the Woke philosophy through 'community organising' long before anyone had heard of it. In those days it still went by its traditional name of Marxism. The reason for the manipulated Trump phenomenon was laid out in Alinsky's 1971 book, *Rules for Radicals*, which was his blueprint for overthrowing democratic and other regimes and replacing them with Sabbatian Marxism. Not surprisingly his to-do list was evident in the Sabbatian French and Russian 'Revolutions' and that in China which will become very relevant in the next chapter about the 'Covid' hoax. Among Alinsky's followers have been the deeply corrupt Barack Obama, House Speaker Nancy Pelosi and Hillary Clinton who described him as a 'hero'. All three are Sabbatian stooges with Pelosi personifying the arrogant corrupt idiocy that so widely fronts up for the Cult inner core. Predictably as a Sabbatian advocate of the 'light-bringer' Alinsky features Lucifer on the dedication page of his book as the original radical who gained

his own kingdom ('Earth' as we shall see). One of Alinsky's golden radical rules was to pick an individual and focus all attention, hatred and blame on them and not to target faceless bureaucracies and corporations. *Rules for Radicals* is really a Sabbatian handbook with its contents repeatedly employed all over the world for centuries and why wouldn't Sabbatians bring to power their designer-villain to be used as the individual on which all attention, hatred and blame was bestowed? This is what they did and the only question for me is how much Trump knew that and how much he was manipulated. A bit of both, I suspect. This was Alinsky's Trump technique from a man who died in 1972. The technique has spanned history:

Pick the target, freeze it, personalize it, polarize it. Don't try to attack abstract corporations or bureaucracies. Identify a responsible individual. Ignore attempts to shift or spread the blame.

From the moment Trump came to illusory power everything was about him. It wasn't about Republican policy or opinion, but all about Trump. Everything he did was presented in negative, derogatory and abusive terms by the Sabbatian-dominated media led by Cult operations such as CNN, MSNBC, *The New York Times* and the Jeff Bezos-owned *Washington Post* – 'Pick the target, freeze it, personalize it, polarize it.' Trump was turned into a demon to be vilified by those who hated him and a demi-god loved by those who worshipped him. This, in turn, had his supporters, too, presented as equally demonic in preparation for the punchline later down the line when Biden was about to take office. It was here's a Trump, there's a Trump, everywhere a Trump, Trump. Virtually every news story or happening was filtered through the lens of 'The Donald'. You loved him or hated him and which one you chose was said to define you as Satan's spawn or a paragon of virtue. Even supporting some Trump policies or statements and not others was enough for an assault on your character. No shades of grey were or are allowed. Everything is black and white (literally and figuratively). A Californian I knew had her head utterly scrambled by her hatred for Trump while telling people they should love each other. She was so totally consumed by

Trump Derangement Syndrome as it became to be known that this glaring contradiction would never have occurred to her. By definition anyone who criticised Trump or praised his opponents was a hero and this lady described Joe Biden as 'a kind, honest gentleman' when he's a provable liar, mega-crook and vicious piece of work to boot. Sabbatians had indeed divided America using Trump as the fall-guy and all along the clock was ticking on the consequences for his supporters.

In hock to his masters

Trump gave Sabbatians via Israel almost everything they wanted in his four years. Ask and you shall receive was the dynamic between himself and Benjamin Netanyahu orchestrated by Trump's ultra-Zionist son-in-law Jared Kushner, his ultra-Zionist Ambassador to Israel, David Friedman, and ultra-Zionist 'Israel adviser', Jason Greenblatt. The last two were central to the running and protecting from collapse of his business empire, the Trump Organisation, and colossal business failures made him forever beholding to Sabbatian networks that bailed him out. By the start of the 1990s Trump owed $4 billion to banks that he couldn't pay and almost $1billion of that was down to him personally and not his companies. This mega-disaster was the result of building two new casinos in Atlantic City and buying the enormous Taj Mahal operation which led to crippling debt payments. He had borrowed fantastic sums from 72 banks with major Sabbatian connections and although the scale of debt should have had him living in a tent alongside the highway they never foreclosed. A plan was devised to lift Trump from the mire by BT Securities Corporation and Rothschild Inc. and the case was handled by Wilber Ross who had worked for the Rothschilds for 27 years. Ross would be named US Commerce Secretary after Trump's election. Another crucial figure in saving Trump was ultra-Zionist 'investor' Carl Icahn who bought the Taj Mahal casino. Icahn was made special economic adviser on financial regulation in the Trump administration. He didn't stay long but still managed to find time to make a tidy sum of a reported $31.3 million when he sold his

holdings affected by the price of steel three days before Trump imposed a 235 percent tariff on steel imports. What amazing bits of luck these people have. Trump and Sabbatian operatives have long had a close association and his mentor and legal adviser from the early 1970s until 1986 was the dark and genetically corrupt ultra-Zionist Roy Cohn who was chief counsel to Senator Joseph McCarthy's 'communist' witch-hunt in the 1950s. *Esquire* magazine published an article about Cohn with the headline 'Don't mess with Roy Cohn'. He was described as the most feared lawyer in New York and 'a ruthless master of dirty tricks ... [with] ... more than one Mafia Don on speed dial'. Cohn's influence, contacts, support and protection made Trump a front man for Sabbatians in New York with their connections to one of Cohn's many criminal employers, the 'Russian' Sabbatian Mafia. Israel-centric media mogul Rupert Murdoch was introduced to Trump by Cohn and they started a long friendship. Cohn died in 1986 weeks after being disbarred for unethical conduct by the Appellate Division of the New York State Supreme Court. The wheels of justice do indeed run slow given the length of Cohn's crooked career.

QAnon-sense

We are asked to believe that Donald Trump with his fundamental connections to Sabbatian networks and operatives has been leading the fight to stop the Sabbatian agenda for the fascistic control of America and the world. Sure he has. A man entrapped during his years in the White House by Sabbatian operatives and whose biggest financial donor was casino billionaire Sheldon Adelson who was Sabbatian to his DNA?? Oh, do come on. Trump has been used to divide America and isolate Pushbackers on the Cult agenda under the heading of 'Trump supporters', 'insurrectionists' and 'white supremacists'. The US Intelligence/Mossad Psyop or psychological operation known as QAnon emerged during the Trump years as a central pillar in the Sabbatian campaign to lead Pushbackers into the trap set by those that wished to destroy them. I knew from the start that QAnon was a scam because I had seen the same scenario many

times before over 30 years under different names and I had written about one in particular in the books. 'Not again' was my reaction when QAnon came to the fore. The same script is pulled out every few years and a new name added to the letterhead. The story always takes the same form: 'Insiders' or 'the good guys' in the government-intelligence-military 'Deep State' apparatus were going to instigate mass arrests of the 'bad guys' which would include the Rockefellers, Rothschilds, Barack Obama, Hillary Clinton, George Soros, etc., etc. Dates are given for when the 'good guys' are going to move in, but the dates pass without incident and new dates are given which pass without incident. The central message to Pushbackers in each case is that they don't have to do anything because there is 'a plan' and it is all going to be sorted by the 'good guys' on the inside. 'Trust the plan' was a QAnon mantra when the only plan was to misdirect Pushbackers into putting their trust in a Psyop they believed to be real. Beware, beware, those who tell you what you want to hear and always check it out. Right up to Biden's inauguration QAnon was still claiming that 'the Storm' was coming and Trump would stay on as president when Biden and his cronies were arrested and jailed. It was never going to happen and of course it didn't, but what did happen as a result provided that punchline to the Sabbatian Trump/QAnon Psyop.

On January 6th, 2021, a very big crowd of Trump supporters gathered in the National Mall in Washington DC down from the Capitol Building to protest at what they believed to be widespread corruption and vote fraud that stopped Trump being re-elected for a second term as president in November, 2020. I say as someone that does not support Trump or Biden that the evidence is clear that major vote-fixing went on to favour Biden, a man with cognitive problems so advanced he can often hardly string a sentence together without reading the words written for him on the Teleprompter. Glaring ballot discrepancies included serious questions about electronic voting machines that make vote rigging a comparative cinch and hundreds of thousands of paper votes that suddenly appeared during already advanced vote counts and virtually all of

them for Biden. Early Trump leads in crucial swing states suddenly began to close and disappear. The pandemic hoax was used as the excuse to issue almost limitless numbers of mail-in ballots with no checks to establish that the recipients were still alive or lived at that address. They were sent to streams of people who had not even asked for them. Private organisations were employed to gather these ballots and who knows what they did with them before they turned up at the counts. The American election system has been manipulated over decades to become a sick joke with more holes than a Swiss cheese for the express purpose of dictating the results. Then there was the criminal manipulation of information by Sabbatian tech giants like Facebook, Twitter and Google-owned YouTube which deleted pro-Trump, anti-Biden accounts and posts while everything in support of Biden was left alone. Sabbatians wanted Biden to win because after the dividing of America it was time for full-on Woke and every aspect of the Cult agenda to be unleashed.

Hunter gatherer

Extreme Silicon Valley bias included blocking information by the *New York Post* exposing a Biden scandal that should have ended his bid for president in the final weeks of the campaign. Hunter Biden, his monumentally corrupt son, is reported to have sent a laptop to be repaired at a local store and failed to return for it. Time passed until the laptop became the property of the store for non-payment of the bill. When the owner saw what was on the hard drive he gave a copy to the FBI who did nothing even though it confirmed widespread corruption in which the Joe Biden family were using his political position, especially when he was vice president to Obama, to make multiple millions in countries around the world and most notably Ukraine and China. Hunter Biden's one-time business partner Tony Bobulinski went public when the story broke in the *New York Post* to confirm the corruption he saw and that Joe Biden not only knew what was going on he also profited from the spoils. Millions were handed over by a Chinese company with close

connections – like all major businesses in China – to the Chinese communist party of President Xi Jinping. Joe Biden even boasted at a meeting of the Cult's World Economic Forum that as vice president he had ordered the government of Ukraine to fire a prosecutor. What he didn't mention was that the same man just happened to be investigating an energy company which was part of Hunter Biden's corrupt portfolio. The company was paying him big bucks for no other reason than the influence his father had. Overnight Biden's presidential campaign should have been over given that he had lied publicly about not knowing what his son was doing. Instead almost the entire Sabbatian-owned mainstream media and Sabbatian-owned Silicon Valley suppressed circulation of the story. This alone went a mighty way to rigging the election of 2020. Cult assets like Mark Zuckerberg at Facebook also spent hundreds of millions to be used in support of Biden and vote 'administration'.

The Cult had used Trump as the focus to divide America and was now desperate to bring in moronic, pliable, corrupt Biden to complete the double-whammy. No way were they going to let little things like the will of the people thwart their plan. Silicon Valley widely censored claims that the election was rigged because it *was* rigged. For the same reason anyone claiming it was rigged was denounced as a 'white supremacist' including the pathetically few Republican politicians willing to say so. Right across the media where the claim was mentioned it was described as a 'false claim' even though these excuses for 'journalists' would have done no research into the subject whatsoever. Trump won seven million more votes than any sitting president had ever achieved while somehow a cognitively-challenged soon to be 78-year-old who was hidden away from the public for most of the campaign managed to win more votes than any presidential candidate in history. It makes no sense. You only had to see election rallies for both candidates to witness the enthusiasm for Trump and the apathy for Biden. Tens of thousands would attend Trump events while Biden was speaking in empty car parks with often only television crews attending and framing their shots to hide the fact that no one was there. It was pathetic to see

footage come to light of Biden standing at a podium making speeches only to TV crews and party fixers while reading the words written for him on massive Teleprompter screens. So, yes, those protestors on January 6th had a point about election rigging, but some were about to walk into a trap laid for them in Washington by the Cult Deep State and its QAnon Psyop. This was the Capitol Hill riot ludicrously dubbed an 'insurrection'.

The spider and the fly

Renegade Minds know there are not two 'sides' in politics, only one side, the Cult, working through all 'sides'. It's a stage show, a puppet show, to direct the perceptions of the population into focusing on diversions like parties and candidates while missing the puppeteers with their hands holding all the strings. The Capitol Hill 'insurrection' brings us back to the Little Big Horn. Having created two distinct opposing groupings – Woke and Pushbackers – the trap was about to be sprung. Pushbackers were to be encircled and isolated by associating them all in the public mind with Trump and then labelling Trump as some sort of Confederate leader. I knew immediately that the Capitol riot was a set-up because of two things. One was how easy the rioters got into the building with virtually no credible resistance and secondly I could see – as with the 'Covid' hoax in the West at the start of 2020 – how the Cult could exploit the situation to move its agenda forward with great speed. My experience of Cult techniques and activities over more than 30 years has showed me that while they do exploit situations they haven't themselves created this never happens with events of fundamental agenda significance. Every time major events giving cultists the excuse to rapidly advance their plan you find they are manipulated into being for the specific reason of providing that excuse – Problem-Reaction-Solution. Only a tiny minority of the huge crowd of Washington protestors sought to gain entry to the Capitol by smashing windows and breaching doors. That didn't matter. The whole crowd and all Pushbackers, even if they did not support Trump, were going to be lumped together as dangerous

insurrectionists and conspiracy theorists. The latter term came into widespread use through a CIA memo in the 1960s aimed at discrediting those questioning the nonsensical official story of the Kennedy assassination and it subsequently became widely employed by the media. It's still being used by inept 'journalists' with no idea of its origin to discredit anyone questioning anything that authority claims to be true. When you are perpetrating a conspiracy you need to discredit the very word itself even though the dictionary definition of conspiracy is merely 'the activity of secretly planning with other people to do something bad or illegal' and 'a general agreement to keep silent about a subject for the purpose of keeping it secret'. On that basis there are conspiracies almost wherever you look. For obvious reasons the Cult and its lapdog media have to claim there are no conspiracies even though the word appears in state laws as with conspiracy to defraud, to murder, and to corrupt public morals.

Agent provocateurs are widely used by the Cult Deep State to manipulate genuine people into acting in ways that suit the desired outcome. By genuine in this case I mean protestors genuinely supporting Trump and claims that the election was stolen. In among them, however, were agents of the state wearing the garb of Trump supporters and QAnon to pump-prime the Capital riot which some genuine Trump supporters naively fell for. I described the situation as 'Come into my parlour said the spider to the fly'. Leaflets appeared through the Woke paramilitary arm Antifa, the anti-fascist fascists, calling on supporters to turn up in Washington looking like Trump supporters even though they hated him. Some of those arrested for breaching the Capitol Building were sourced to Antifa and its stable mate Black Lives Matter. Both organisations are funded by Cult billionaires and corporations. One man charged for the riot was according to his lawyer a former FBI agent who had held top secret security clearance for 40 years. Attorney Thomas Plofchan said of his client, 66-year-old Thomas Edward Caldwell:

He has held a Top Secret Security Clearance since 1979 and has undergone multiple Special Background Investigations in support of his clearances. After retiring from the Navy, he

worked as a section chief for the Federal Bureau of Investigation from 2009-2010 as a GS-12 [mid-level employee].

He also formed and operated a consulting firm performing work, often classified, for U.S government customers including the US. Drug Enforcement Agency, Department of Housing and Urban Development, the US Coast Guard, and the US Army Personnel Command.

A judge later released Caldwell pending trial in the absence of evidence about a conspiracy or that he tried to force his way into the building. *The New York Post* reported a 'law enforcement source' as saying that 'at least two known Antifa members were spotted' on camera among Trump supporters during the riot while one of the rioters arrested was John Earle Sullivan, a seriously extreme Black Lives Matter Trump-hater from Utah who was previously arrested and charged in July, 2020, over a BLM-Antifa riot in which drivers were threatened and one was shot. Sullivan is the founder of Utah-based Insurgence USA which is an affiliate of the Cult-created-and-funded Black Lives Matter movement. Footage appeared and was then deleted by Twitter of Trump supporters calling out Antifa infiltrators and a group was filmed changing into pro-Trump clothing before the riot. Security at the building was *pathetic* – as planned. Colonel Leroy Fletcher Prouty, a man with long experience in covert operations working with the US security apparatus, once described the tell-tale sign to identify who is involved in an assassination. He said:

No one has to direct an assassination – it happens. The active role is played secretly by permitting it to happen. This is the greatest single clue. Who has the power to call off or reduce the usual security precautions?

This principle applies to many other situations and certainly to the Capitol riot of January 6th, 2021.

The sting

With such a big and potentially angry crowd known to be gathering near the Capitol the security apparatus would have had a major police detail to defend the building with National Guard troops on

standby given the strength of feeling among people arriving from all over America encouraged by the QAnon Psyop and statements by Donald Trump. Instead Capitol Police 'security' was flimsy, weak, and easily breached. The same number of officers was deployed as on a regular day and that is a blatant red flag. They were not staffed or equipped for a possible riot that had been an obvious possibility in the circumstances. No protective and effective fencing worth the name was put in place and there were no contingency plans. The whole thing was basically a case of standing aside and waving people in. Once inside police mostly backed off apart from one Capitol police officer who ridiculously shot dead unarmed Air Force veteran protestor Ashli Babbitt without a warning as she climbed through a broken window. The 'investigation' refused to name or charge the officer after what must surely be considered a murder in the circumstances. They just lifted a carpet and swept. The story was endlessly repeated about five people dying in the 'armed insurrection' when there was no report of rioters using weapons. Apart from Babbitt the other four died from a heart attack, strokes and apparently a drug overdose. Capitol police officer Brian Sicknick was reported to have died after being bludgeoned with a fire extinguisher when he was alive after the riot was over and died later of what the Washington Medical Examiner's Office said was a stroke. Sicknick had no external injuries. The lies were delivered like rapid fire. There was a narrative to build with incessant repetition of the lie until the lie became the accepted 'everybody knows that' truth. The 'Big Lie' technique of Nazi Propaganda Minister Joseph Goebbels is constantly used by the Cult which was behind the Nazis and is today behind the 'Covid' and 'climate change' hoaxes. Goebbels said:

If you tell a lie big enough and keep repeating it, people will eventually come to believe it. The lie can be maintained only for such time as the State can shield the people from the political, economic and/or military consequences of the lie. It thus becomes vitally important for the State to use all of its powers to repress dissent, for the truth is the mortal enemy of the lie, and thus by extension, the truth is the greatest enemy of the State.

Most protestors had a free run of the Capitol Building. This allowed pictures to be taken of rioters in iconic parts of the building including the Senate chamber which could be used as propaganda images against all Pushbackers. One Congresswoman described the scene as 'the worst kind of non-security anybody could ever imagine'. Well, the first part was true, but someone obviously did imagine it and made sure it happened. Some photographs most widely circulated featured people wearing QAnon symbols and now the Psyop would be used to dub all QAnon followers with the ubiquitous fit-all label of 'white supremacist' and 'insurrectionists'. When a Muslim extremist called Noah Green drove his car at two police officers at the Capitol Building killing one in April, 2021, there was no such political and media hysteria. They were just disappointed he wasn't white.

The witch-hunt

Government prosecutor Michael Sherwin, an aggressive, dark-eyed, professional Rottweiler led the 'investigation' and to call it over the top would be to understate reality a thousand fold. Hundreds were tracked down and arrested for the crime of having the wrong political views and people were jailed who had done nothing more than walk in the building, committed no violence or damage to property, took a few pictures and left. They were labelled a 'threat to the Republic' while Biden sat in the White House signing executive orders written for him that were dismantling 'the Republic'. Even when judges ruled that a mother and son should not be in jail the government kept them there. Some of those arrested have been badly beaten by prison guards in Washington and lawyers for one man said he suffered a fractured skull and was made blind in one eye. Meanwhile a woman is shot dead for no reason by a Capitol Police officer and we are not allowed to know who he is never mind what has happened to him although that will be *nothing*. The Cult's QAnon/Trump sting to identify and isolate Pushbackers and then target them on the road to crushing and deleting them was a resounding success. You would have thought the Russians had

invaded the building at gunpoint and lined up senators for a firing squad to see the political and media reaction. Congresswoman Alexandria Ocasio-Cortez is a child in a woman's body, a terrible-twos, me, me, me, Woker narcissist of such proportions that words have no meaning. She said she thought she was going to die when 'insurrectionists' banged on her office door. It turned out she wasn't even in the Capitol Building when the riot was happening and the 'banging' was a Capitol Police officer. She referred to herself as a 'survivor' which is an insult to all those true survivors of violent and sexual abuse while she lives her pampered and privileged life talking drivel for a living. Her Woke colleague and fellow mega-narcissist Rashida Tlaib broke down describing the devastating effect on her, too, of *not being* in the building when the rioters were there. Ocasio-Cortez and Tlaib are members of a fully-Woke group of Congresswomen known as 'The Squad' along with Ilhan Omar and Ayanna Pressley. The Squad from what I can see can be identified by its vehement anti-white racism, anti-white men agenda, and, as always in these cases, the absence of brain cells on active duty.

The usual suspects were on the riot case immediately in the form of Democrat ultra-Zionist senators and operatives Chuck Schumer and Adam Schiff demanding that Trump be impeached for 'his part in the insurrection'. The same pair of prats had led the failed impeachment of Trump over the invented 'Russia collusion' nonsense which claimed Russia had helped Trump win the 2016 election. I didn't realise that Tel Aviv had been relocated just outside Moscow. I must find an up-to-date map. The Russia hoax was a Sabbatian operation to keep Trump occupied and impotent and to stop any rapport with Russia which the Cult wants to retain as a perceptual enemy to be pulled out at will. Puppet Biden began attacking Russia when he came to office as the Cult seeks more upheaval, division and war across the world. A two-year stage show 'Russia collusion inquiry' headed by the not-very-bright former 9/11 FBI chief Robert Mueller, with support from 19 lawyers, 40 FBI agents plus intelligence analysts, forensic accountants and other

staff, devoured tens of millions of dollars and found no evidence of Russia collusion which a ten-year-old could have told them on day one. Now the same moronic Schumer and Schiff wanted a second impeachment of Trump over the Capitol 'insurrection' (riot) which the arrested development of Schumer called another 'Pearl Harbor' while others compared it with 9/11 in which 3,000 died and, in the case of CNN, with the Rwandan genocide in the 1990s in which an estimated 500,000 to 600,000 were murdered, between 250, 000 and 500,000 women were raped, and populations of whole towns were hacked to death with machetes. To make those comparisons purely for Cult political reasons is beyond insulting to those that suffered and lost their lives and confirms yet again the callous inhumanity that we are dealing with. Schumer is a monumental idiot and so is Schiff, but they serve the Cult agenda and do whatever they're told so they get looked after. Talking of idiots – another inane man who spanned the Russia and Capitol impeachment attempts was Senator Eric Swalwell who had the nerve to accuse Trump of collusion with the Russians while sleeping with a Chinese spy called Christine Fang or 'Fang Fang' which is straight out of a Bond film no doubt starring Klaus Schwab as the bloke living on a secret island and controlling laser weapons positioned in space and pointing at world capitals. Fang Fang plays the part of Bond's infiltrator girlfriend which I'm sure she would enjoy rather more than sharing a bed with the brainless Swalwell, lying back and thinking of China. The FBI eventually warned Swalwell about Fang Fang which gave her time to escape back to the Chinese dictatorship. How very thoughtful of them. The second Trump impeachment also failed and hardly surprising when an impeachment is supposed to remove a sitting president and by the time it happened Trump was no longer president. These people are running your country America, well, officially anyway. Terrifying isn't it?

Outcomes tell the story - always

The outcome of all this – and it's the *outcome* on which Renegade Minds focus, not the words – was that a vicious, hysterical and

obviously pre-planned assault was launched on Pushbackers to censor, silence and discredit them and even targeted their right to earn a living. They have since been condemned as 'domestic terrorists' that need to be treated like Al-Qaeda and Islamic State. 'Domestic terrorists' is a label the Cult has been trying to make stick since the period of the Oklahoma bombing in 1995 which was blamed on 'far-right domestic terrorists'. If you read *The Trigger* you will see that the bombing was clearly a Problem-Reaction-Solution carried out by the Deep State during a Bill Clinton administration so corrupt that no dictionary definition of the term would even nearly suffice. Nearly 30, 000 troops were deployed from all over America to the empty streets of Washington for Biden's inauguration. Ten thousand of them stayed on with the pretext of protecting the capital from insurrectionists when it was more psychological programming to normalise the use of the military in domestic law enforcement in support of the Cult plan for a police-military state. Biden's fascist administration began a purge of 'wrong-thinkers' in the military which means anyone that is not on board with Woke. The Capitol Building was surrounded by a fence with razor wire and the Land of the Free was further symbolically and literally dismantled. The circle was completed with the installation of Biden and the exploitation of the QAnon Psyop.

America had never been so divided since the civil war of the 19th century, Pushbackers were isolated and dubbed terrorists and now, as was always going to happen, the Cult immediately set about deleting what little was left of freedom and transforming American society through a swish of the hand of the most controlled 'president' in American history leading (officially at least) the most extreme regime since the country was declared an independent state on July 4th, 1776. Biden issued undebated, dictatorial executive orders almost by the hour in his opening days in office across the whole spectrum of the Cult wish-list including diluting controls on the border with Mexico allowing thousands of migrants to illegally enter the United States to transform the demographics of America and import an election-changing number of perceived Democrat

voters. Then there were Biden deportation amnesties for the already illegally resident (estimated to be as high as 20 or even 30 million). A bill before Congress awarded American citizenship to anyone who could prove they had worked in agriculture for just 180 days in the previous two years as 'Big Ag' secured its slave labour long-term. There were the plans to add new states to the union such as Puerto Rico and making Washington DC a state. They are all parts of a plan to ensure that the Cult-owned Woke Democrats would be permanently in power.

Border – what border?

I have exposed in detail in other books how mass immigration into the United States and Europe is the work of Cult networks fuelled by the tens of billions spent to this and other ends by George Soros and his global Open Society (open borders) Foundations. The impact can be seen in America alone where the population has increased by *100 million* in little more than 30 years mostly through immigration. I wrote in *The Answer* that the plan was to have so many people crossing the southern border that the numbers become unstoppable and we are now there under Cult-owned Biden. El Salvador in Central America puts the scale of what is happening into context. A third of the population now lives in the United States, much of it illegally, and many more are on the way. The methodology is to crush Central and South American countries economically and spread violence through machete-wielding psychopathic gangs like MS-13 based in El Salvador and now operating in many American cities. Biden-imposed lax security at the southern border means that it is all but open. He said before his 'election' that he wanted to see a surge towards the border if he became president and that was the green light for people to do just that after election day to create the human disaster that followed for both America and the migrants. When that surge came the imbecilic Alexandria Ocasio-Cortez said it wasn't a 'surge' because they are 'children, not insurgents' and the term 'surge' (used by Biden) was a claim of 'white supremacists'.

This disingenuous lady may one day enter the realm of the most basic intelligence, but it won't be any time soon.

Sabbatians and the Cult are in the process of destroying America by importing violent people and gangs in among the genuine to terrorise American cities and by overwhelming services that cannot cope with the sheer volume of new arrivals. Something similar is happening in Europe as Western society in general is targeted for demographic and cultural transformation and upheaval. The plan demands violence and crime to create an environment of intimidation, fear and division and Soros has been funding the election of district attorneys across America who then stop prosecuting many crimes, reduce sentences for violent crimes and free as many violent criminals as they can. Sabbatians are creating the chaos from which order – their order – can respond in a classic Problem-Reaction-Solution. A Freemasonic moto says 'Ordo Ab Chao' (Order out of Chaos) and this is why the Cult is constantly creating chaos to impose a new 'order'. Here you have the reason the Cult is constantly creating chaos. The 'Covid' hoax can be seen with those entering the United States by plane being forced to take a 'Covid' test while migrants flooding through southern border processing facilities do not. Nothing is put in the way of mass migration and if that means ignoring the government's own 'Covid' rules then so be it. They know it's all bullshit anyway. Any pushback on this is denounced as 'racist' by Wokers and Sabbatian fronts like the ultra-Zionist Anti-Defamation League headed by the appalling Jonathan Greenblatt which at the same time argues that Israel should not give citizenship and voting rights to more Palestinian Arabs or the 'Jewish population' (in truth the Sabbatian network) will lose control of the country.

Society-changing numbers

Biden's masters have declared that countries like El Salvador are so dangerous that their people must be allowed into the United States for humanitarian reasons when there are fewer murders in large parts of many Central American countries than in US cities like

Baltimore. That is not to say Central America cannot be a dangerous place and Cult-controlled American governments have been making it so since way back, along with the dismantling of economies, in a long-term plan to drive people north into the United States. Parts of Central America are very dangerous, but in other areas the story is being greatly exaggerated to justify relaxing immigration criteria. Migrants are being offered free healthcare and education in the United States as another incentive to head for the border and there is no requirement to be financially independent before you can enter to prevent the resources of America being drained. You can't blame migrants for seeking what they believe will be a better life, but they are being played by the Cult for dark and nefarious ends. The numbers since Biden took office are huge. In February, 2021, more than 100,000 people were known to have tried to enter the US illegally through the southern border (it was 34,000 in the same month in 2020) and in March it was 170,000 – a 418 percent increase on March, 2020. These numbers are only known people, not the ones who get in unseen. The true figure for migrants illegally crossing the border in a single month was estimated by one congressman at 250,000 and that number will only rise under Biden's current policy. Gangs of murdering drug-running thugs that control the Mexican side of the border demand money – thousands of dollars – to let migrants cross the Rio Grande into America. At the same time gun battles are breaking out on the border several times a week between rival Mexican drug gangs (which now operate globally) who are equipped with sophisticated military-grade weapons, grenades and armoured vehicles. While the Capitol Building was being 'protected' from a non-existent 'threat' by thousands of troops, and others were still deployed at the time in the Cult Neocon war in Afghanistan, the southern border of America was left to its fate. This is not incompetence, it is cold calculation.

By March, 2021, there were 17,000 unaccompanied children held at border facilities and many of them are ensnared by people traffickers for paedophile rings and raped on their journey north to America. This is not conjecture – this is fact. Many of those designated

children are in reality teenage boys or older. Meanwhile Wokers posture their self-purity for encouraging poor and tragic people to come to America and face this nightmare both on the journey and at the border with the disgusting figure of House Speaker Nancy Pelosi giving disingenuous speeches about caring for migrants. The woman's evil. Wokers condemned Trump for having children in cages at the border (so did Obama, *Shhhh*), but now they are sleeping on the floor without access to a shower with one border facility 729 percent over capacity. The Biden insanity even proposed flying migrants from the southern border to the northern border with Canada for 'processing'. The whole shambles is being overseen by ultra-Zionist Secretary of Homeland Security, the moronic liar Alejandro Mayorkas, who banned news cameras at border facilities to stop Americans seeing what was happening. Mayorkas said there was not a ban on news crews; it was just that they were not allowed to film. Alongside him at Homeland Security is another ultra-Zionist Cass Sunstein appointed by Biden to oversee new immigration laws. Sunstein despises conspiracy researchers to the point where he suggests they should be banned or *taxed* for having such views. The man is not bonkers or anything. He's perfectly well-adjusted, but adjusted to what is the question. Criticise what is happening and you are a 'white supremacist' when earlier non-white immigrants also oppose the numbers which effect their lives and opportunities. Black people in poor areas are particularly damaged by uncontrolled immigration and the increased competition for work opportunities with those who will work for less. They are also losing voting power as Hispanics become more dominant in former black areas. It's a downward spiral for them while the billionaires behind the policy drone on about how much they care about black people and 'racism'. None of this is about compassion for migrants or black people – that's just wind and air. Migrants are instead being mercilessly exploited to transform America while the countries they leave are losing their future and the same is true in Europe. Mass immigration may now be the work of Woke Democrats, but it can be traced back to the 1986 Immigration Reform and Control Act (it

wasn't) signed into law by Republican hero President Ronald Reagan which gave amnesty to millions living in the United States illegally and other incentives for people to head for the southern border. Here we have the one-party state at work again.

Save me syndrome

Almost every aspect of what I have been exposing as the Cult agenda was on display in even the first days of 'Biden' with silencing of Pushbackers at the forefront of everything. A Renegade Mind will view the Trump years and QAnon in a very different light to their supporters and advocates as the dots are connected. The QAnon/Trump Psyop has given the Cult all it was looking for. We may not know how much, or little, that Trump realised he was being used, but that's a side issue. This pincer movement produced the desired outcome of dividing America and having Pushbackers isolated. To turn this around we have to look at new routes to empowerment which do not include handing our power to other people and groups through what I will call the 'Save Me Syndrome' – 'I want someone else to do it so that I don't have to'. We have seen this at work throughout human history and the QAnon/Trump Psyop is only the latest incarnation alongside all the others. Religion is an obvious expression of this when people look to a 'god' or priest to save them or tell them how to be saved and then there are 'save me' politicians like Trump. Politics is a diversion and not a 'saviour'. It is a means to block positive change, not make it possible.

Save Me Syndrome always comes with the same repeating theme of handing your power to whom or what you believe will save you while your real 'saviour' stares back from the mirror every morning. Renegade Minds are constantly vigilant in this regard and always asking the question 'What can I do?' rather than 'What can someone else do for me?' Gandhi was right when he said: 'You must be the change you want to see in the world.' We are indeed the people we have been waiting for. We are presented with a constant raft of reasons to concede that power to others and forget where the real power is. Humanity has the numbers and the Cult does not. It has to

use diversion and division to target the unstoppable power that comes from unity. Religions, governments, politicians, corporations, media, QAnon, are all different manifestations of this power-diversion and dilution. Refusing to give your power to governments and instead handing it to Trump and QAnon is not to take a new direction, but merely to recycle the old one with new names on the posters. I will explore this phenomenon as we proceed and how to break the cycles and recycles that got us here through the mists of repeating perception and so repeating history.

For now we shall turn to the most potent example in the entire human story of the consequences that follow when you give your power away. I am talking, of course, of the 'Covid' hoax.

CHAPTER FOUR

'Covid': Calculated catastrophe

Facts are threatening to those invested in fraud
DaShanne Stokes

We can easily unravel the real reason for the 'Covid pandemic' hoax by employing the Renegade Mind methodology that I have outlined this far. We'll start by comparing the long-planned Cult outcome with the 'Covid pandemic' outcome. Know the outcome and you'll see the journey.

I have highlighted the plan for the Hunger Games Society which has been in my books for so many years with the very few controlling the very many through ongoing dependency. To create this dependency it is essential to destroy independent livelihoods, businesses and employment to make the population reliant on the state (the Cult) for even the basics of life through a guaranteed pittance income. While independence of income remained these Cult ambitions would be thwarted. With this knowledge it was easy to see where the 'pandemic' hoax was going once talk of 'lockdowns' began and the closing of all but perceived 'essential' businesses to 'save' us from an alleged 'deadly virus'. Cult corporations like Amazon and Walmart were naturally considered 'essential' while mom and pop shops and stores had their doors closed by fascist decree. As a result with every new lockdown and new regulation more small and medium, even large businesses not owned by the Cult, went to the wall while Cult giants and their frontmen and women grew financially fatter by the second. Mom and pop were

denied an income and the right to earn a living and the wealth of people like Jeff Bezos (Amazon), Mark Zuckerberg (Facebook) and Sergei Brin and Larry Page (Google/Alphabet) have reached record levels. The Cult was increasing its own power through further dramatic concentrations of wealth while the competition was being destroyed and brought into a state of dependency. Lockdowns have been instigated to secure that very end and were never anything to do with health. My brother Paul spent 45 years building up a bus repair business, but lockdowns meant buses were running at a fraction of normal levels for months on end. Similar stories can told in their hundreds of millions worldwide. Efforts of a lifetime coldly destroyed by Cult multi-billionaires and their lackeys in government and law enforcement who continued to earn their living from the taxation of the people while denying the right of the same people to earn theirs. How different it would have been if those making and enforcing these decisions had to face the same financial hardships of those they affected, but they never do.

Gates of Hell

Behind it all in the full knowledge of what he is doing and why is the psychopathic figure of Cult operative Bill Gates. His puppet Tedros at the World Health Organization declared 'Covid' a pandemic in March, 2020. The WHO had changed the definition of a 'pandemic' in 2009 just a month before declaring the 'swine flu pandemic' which would not have been so under the previous definition. The same applies to 'Covid'. The definition had included… 'an infection by an infectious agent, occurring simultaneously in different countries, with a significant mortality rate relative to the proportion of the population infected'. The new definition removed the need for 'significant mortality'. The 'pandemic' has been fraudulent even down to the definition, but Gates demanded economy-destroying lockdowns, school closures, social distancing, mandatory masks, a 'vaccination' for every man, woman and child on the planet and severe consequences and restrictions for those that refused. Who gave him this power? The

Cult did which he serves like a little boy in short trousers doing what his daddy tells him. He and his psychopathic missus even smiled when they said that much worse was to come (what they knew was planned to come). Gates responded in the matter-of-fact way of all psychopaths to a question about the effect on the world economy of what he was doing:

> Well, it won't go to zero but it will shrink. Global GDP is probably going to take the biggest hit ever [Gates was smiling as he said this] … in my lifetime this will be the greatest economic hit. But you don't have a choice. People act as if you have a choice. People don't feel like going to the stadium when they might get infected … People are deeply affected by seeing these stats, by knowing they could be part of the transmission chain, old people, their parents and grandparents, could be affected by this, and so you don't get to say ignore what is going on here.
>
> There will be the ability to open up, particularly in rich countries, if things are done well over the next few months, but for the world at large normalcy only returns when we have largely vaccinated the entire population.

The man has no compassion or empathy. How could he when he's a psychopath like all Cult players? My own view is that even beyond that he is very seriously mentally ill. Look in his eyes and you can see this along with his crazy flailing arms. You don't do what he has done to the world population since the start of 2020 unless you are mentally ill and at the most extreme end of psychopathic. You especially don't do it when to you know, as we shall see, that cases and deaths from 'Covid' are fakery and a product of monumental figure massaging. 'These stats' that Gates referred to are based on a 'test' that's not testing for the 'virus' as he has known all along. He made his fortune with big Cult support as an infamously ruthless software salesman and now buys global control of 'health' (death) policy without the population he affects having any say. It's a breathtaking outrage. Gates talked about people being deeply affected by fear of 'Covid' when that was because of *him* and his global network lying to them minute-by-minute supported by a lying media that he seriously influences and funds to the tune of hundreds of millions. He's handed big sums to media operations including the BBC, NBC, Al Jazeera, Univision, *PBS NewsHour*,

ProPublica, National Journal, The Guardian, The Financial Times, The Atlantic, Texas Tribune, USA Today publisher Gannett, *Washington Monthly, Le Monde,* Center for Investigative Reporting, Pulitzer Center on Crisis Reporting, National Press Foundation, International Center for Journalists, Solutions Journalism Network, the Poynter Institute for Media Studies, and many more. Gates is everywhere in the 'Covid' hoax and the man must go to prison – or a mental facility – for the rest of his life and his money distributed to those he has taken such enormous psychopathic pleasure in crushing.

The Muscle

The Hunger Games global structure demands a police-military state – a fusion of the two into one force – which viciously imposes the will of the Cult on the population and protects the Cult from public rebellion. In that regard, too, the 'Covid' hoax just keeps on giving. Often unlawful, ridiculous and contradictory 'Covid' rules and regulations have been policed across the world by moronic automatons and psychopaths made faceless by face-nappy masks and acting like the Nazi SS and fascist blackshirts and brownshirts of Hitler and Mussolini. The smallest departure from the rules decreed by the psychos in government and their clueless gofers were jumped upon by the face-nappy fascists. Brutality against public protestors soon became commonplace even on girls, women and old people as the brave men with the batons – the Face-Nappies as I call them – broke up peaceful protests and handed out fines like confetti to people who couldn't earn a living let alone pay hundreds of pounds for what was once an accepted human right. Robot Face-Nappies of Nottingham police in the English East Midlands fined one group £11,000 for attending a child's birthday party. For decades I charted the transformation of law enforcement as genuine, decent officers were replaced with psychopaths and the brain dead who would happily and brutally do whatever their masters told them. Now they were let loose on the public and I would emphasise the point that none of this just happened. The step-by-step change in the dynamic between police and public was orchestrated from the shadows by

those who knew where this was all going and the same with the perceptual reframing of those in all levels of authority and official administration through 'training courses' by organisations such as Common Purpose which was created in the late 1980s and given a massive boost in Blair era Britain until it became a global phenomenon. Supposed public 'servants' began to view the population as the enemy and the same was true of the police. This was the start of the explosion of behaviour manipulation organisations and networks preparing for the all-war on the human psyche unleashed with the dawn of 2020. I will go into more detail about this later in the book because it is a core part of what is happening.

Police desecrated beauty spots to deter people gathering and arrested women for walking in the countryside alone 'too far' from their homes. We had arrogant, clueless sergeants in the Isle of Wight police where I live posting on Facebook what they insisted the population must do or else. A schoolmaster sergeant called Radford looked young enough for me to ask if his mother knew he was out, but he was posting what he *expected* people to do while a Sergeant Wilkinson boasted about fining lads for meeting in a McDonald's car park where they went to get a lockdown takeaway. Wilkinson added that he had even cancelled their order. What a pair of prats these people are and yet they have increasingly become the norm among Jackboot Johnson's Yellowshirts once known as the British police. This was the theme all over the world with police savagery common during lockdown protests in the United States, the Netherlands, and the fascist state of Victoria in Australia under its tyrannical and again moronic premier Daniel Andrews. Amazing how tyrannical and moronic tend to work as a team and the same combination could be seen across America as arrogant, narcissistic Woke governors and mayors such as Gavin Newsom (California), Andrew Cuomo (New York), Gretchen Whitmer (Michigan), Lori Lightfoot (Chicago) and Eric Garcetti (Los Angeles) did their Nazi and Stalin impressions with the full support of the compliant brutality of their enforcers in uniform as they arrested small business owners defying

fascist shutdown orders and took them to jail in ankle shackles and handcuffs. This happened to bistro owner Marlena Pavlos-Hackney in Gretchen Whitmer's fascist state of Michigan when police arrived to enforce an order by a state-owned judge for 'putting the community at risk' at a time when other states like Texas were dropping restrictions and migrants were pouring across the southern border without any 'Covid' questions at all. I'm sure there are many officers appalled by what they are ordered to do, but not nearly enough of them. If they were truly appalled they would not do it. As the months passed every opportunity was taken to have the military involved to make their presence on the streets ever more familiar and 'normal' for the longer-term goal of police-military fusion.

Another crucial element to the Hunger Games enforcement network has been encouraging the public to report neighbours and others for 'breaking the lockdown rules'. The group faced with £11,000 in fines at the child's birthday party would have been dobbed-in by a neighbour with a brain the size of a pea. The technique was most famously employed by the Stasi secret police in communist East Germany who had public informants placed throughout the population. A police chief in the UK says his force doesn't need to carry out 'Covid' patrols when they are flooded with so many calls from the public reporting other people for visiting the beach. Dorset police chief James Vaughan said people were so enthusiastic about snitching on their fellow humans they were now operating as an auxiliary arm of the police: 'We are still getting around 400 reports a week from the public, so we will respond to reports …We won't need to be doing hotspot patrols because people are very quick to pick the phone up and tell us.' Vaughan didn't say that this is a pillar of all tyrannies of whatever complexion and the means to hugely extend the reach of enforcement while spreading distrust among the people and making them wary of doing anything that might get them reported. Those narcissistic Isle of Wight sergeants Radford and Wilkinson never fail to add a link to their Facebook posts where the public can inform on their fellow slaves.

Neither would be self-aware enough to realise they were imitating the Stasi which they might well never have heard of. Government psychologists that I will expose later laid out a policy to turn communities against each other in the same way.

A coincidence? Yep, and I can knit fog

I knew from the start of the alleged pandemic that this was a Cult operation. It presented limitless potential to rapidly advance the Cult agenda and exploit manipulated fear to demand that every man, woman and child on the planet was 'vaccinated' in a process never used on humans before which infuses self-replicating *synthetic* material into human cells. Remember the plan to transform the human body from a biological to a synthetic biological state. I'll deal with the 'vaccine' (that's not actually a vaccine) when I focus on the genetic agenda. Enough to say here that mass global 'vaccination' justified by this 'new virus' set alarms ringing after 30 years of tracking these people and their methods. The 'Covid' hoax officially beginning in China was also a big red flag for reasons I will be explaining. The agenda potential was so enormous that I could dismiss any idea that the 'virus' appeared naturally. Major happenings with major agenda implications never occur without Cult involvement in making them happen. My questions were twofold in early 2020 as the media began its campaign to induce global fear and hysteria: Was this alleged infectious agent released on purpose by the Cult or did it even exist at all? I then did what I always do in these situations. I sat, observed and waited to see where the evidence and information would take me. By March and early April synchronicity was strongly – and ever more so since then – pointing me in the direction of *there is no 'virus'*. I went public on that with derision even from swathes of the alternative media that voiced a scenario that the Chinese government released the 'virus' in league with Deep State elements in the United States from a top-level bio-lab in Wuhan where the 'virus' is said to have first appeared. I looked at that possibility, but I didn't buy it for several reasons. Deaths from the 'virus' did not in any way match what they

would have been with a 'deadly bioweapon' and it is much more effective if you sell the *illusion* of an infectious agent rather than having a real one unless you can control through injection who has it and who doesn't. Otherwise you lose control of events. A made-up 'virus' gives you a blank sheet of paper on which you can make it do whatever you like and have any symptoms or mutant 'variants' you choose to add while a real infectious agent would limit you to what it actually does. A phantom disease allows you to have endless ludicrous 'studies' on the 'Covid' dollar to widen the perceived impact by inventing ever more 'at risk' groups including one study which said those who walk slowly may be almost four times more likely to die from the 'virus'. People are in psychiatric wards for less.

A real 'deadly bioweapon' can take out people in the hierarchy that are not part of the Cult, but essential to its operation. Obviously they don't want that. Releasing a real disease means you immediately lose control of it. Releasing an illusory one means you don't. Again it's vital that people are extra careful when dealing with what they want to hear. A bioweapon unleashed from a Chinese laboratory in collusion with the American Deep State may fit a conspiracy narrative, but is it true? Would it not be far more effective to use the excuse of a 'virus' to justify the real bioweapon – the 'vaccine'? That way your disease agent does not have to be transmitted and arrives directly through a syringe. I saw a French virologist Luc Montagnier quoted in the alternative media as saying he had discovered that the alleged 'new' severe acute respiratory syndrome coronavirus , or SARS-CoV-2, was made artificially and included elements of the human immunodeficiency 'virus' (HIV) and a parasite that causes malaria. SARS-CoV-2 is alleged to trigger an alleged illness called Covid-19. I remembered Montagnier's name from my research years before into claims that an HIV 'retrovirus' causes AIDs – claims that were demolished by Berkeley virologist Peter Duesberg who showed that no one had ever proved that HIV causes acquired immunodeficiency syndrome or AIDS. Claims that become accepted as fact, publicly and medically, with no proof whatsoever are an ever-recurring story that profoundly applies to

'Covid'. Nevertheless, despite the lack of proof, Montagnier's team at the Pasteur Institute in Paris had a long dispute with American researcher Robert Gallo over which of them discovered and isolated the HIV 'virus' and with *no evidence* found it to cause AIDS. You will see later that there is also no evidence that any 'virus' causes any disease or that there is even such a thing as a 'virus' in the way it is said to exist. The claim to have 'isolated' the HIV 'virus' will be presented in its real context as we come to the shocking story – and it is a story – of SARS-CoV-2 and so will Montagnier's assertion that he identified the full SARS-CoV-2 genome.

Hoax in the making

We can pick up the 'Covid' story in 2010 and the publication by the Rockefeller Foundation of a document called 'Scenarios for the Future of Technology and International Development'. The inner circle of the Rockefeller family has been serving the Cult since John D. Rockefeller (1839-1937) made his fortune with Standard Oil. It is less well known that the same Rockefeller – the Bill Gates of his day – was responsible for establishing what is now referred to as 'Big Pharma', the global network of pharmaceutical companies that make outrageous profits dispensing scalpel and drug 'medicine' and are obsessed with pumping vaccines in ever-increasing number into as many human arms and backsides as possible. John D. Rockefeller was the driving force behind the creation of the 'education' system in the United States and elsewhere specifically designed to program the perceptions of generations thereafter. The Rockefeller family donated exceptionally valuable land in New York for the United Nations building and were central in establishing the World Health Organization in 1948 as an agency of the UN which was created from the start as a Trojan horse and stalking horse for world government. Now enter Bill Gates. His family and the Rockefellers have long been extremely close and I have seen genealogy which claims that if you go back far enough the two families fuse into the same bloodline. Gates has said that the Bill and Melinda Gates Foundation was inspired by the Rockefeller Foundation and why not

when both are serving the same Cult? Major tax-exempt foundations are overwhelmingly criminal enterprises in which Cult assets fund the Cult agenda in the guise of 'philanthropy' while avoiding tax in the process. Cult operatives can become mega-rich in their role of front men and women for the psychopaths at the inner core and they, too, have to be psychopaths to knowingly serve such evil. Part of the deal is that a big percentage of the wealth gleaned from representing the Cult has to be spent advancing the ambitions of the Cult and hence you have the Rockefeller Foundation, Bill and Melinda Gates Foundation (and *so* many more) and people like George Soros with his global Open Society Foundations spending their billions in pursuit of global Cult control. Gates is a global public face of the Cult with his interventions in world affairs including Big Tech influence; a central role in the 'Covid' and 'vaccine' scam; promotion of the climate change shakedown; manipulation of education; geoengineering of the skies; and his food-control agenda as the biggest owner of farmland in America, his GMO promotion and through other means. As one writer said: 'Gates monopolizes or wields disproportionate influence over the tech industry, global health and vaccines, agriculture and food policy (including biopiracy and fake food), weather modification and other climate technologies, surveillance, education and media.' The almost limitless wealth secured through Microsoft and other not-allowed-to-fail ventures (including vaccines) has been ploughed into a long, long list of Cult projects designed to enslave the entire human race. Gates and the Rockefellers have been working as one unit with the Rockefeller-established World Health Organization leading global 'Covid' policy controlled by Gates through his mouth-piece Tedros. Gates became the WHO's biggest funder when Trump announced that the American government would cease its donations, but Biden immediately said he would restore the money when he took office in January, 2021. The Gates Foundation (the Cult) owns through limitless funding the world health system and the major players across the globe in the 'Covid' hoax.

Okay, with that background we return to that Rockefeller Foundation document of 2010 headed 'Scenarios for the Future of Technology and International Development' and its 'imaginary' epidemic of a virulent and deadly influenza strain which infected 20 percent of the global population and killed eight million in seven months. The Rockefeller scenario was that the epidemic destroyed economies, closed shops, offices and other businesses and led to governments imposing fierce rules and restrictions that included mandatory wearing of face masks and body-temperature checks to enter communal spaces like railway stations and supermarkets. The document predicted that even after the height of the Rockefeller-envisaged epidemic the authoritarian rule would continue to deal with further pandemics, transnational terrorism, environmental crises and rising poverty. Now you may think that the Rockefellers are our modern-day seers or alternatively, and rather more likely, that they well knew what was planned a few years further on. Fascism had to be imposed, you see, to 'protect citizens from risk and exposure'. The Rockefeller scenario document said:

During the pandemic, national leaders around the world flexed their authority and imposed airtight rules and restrictions, from the mandatory wearing of face masks to body-temperature checks at the entries to communal spaces like train stations and supermarkets. Even after the pandemic faded, this more authoritarian control and oversight of citizens and their activities stuck and even intensified. In order to protect themselves from the spread of increasingly global problems – from pandemics and transnational terrorism to environmental crises and rising poverty – leaders around the world took a firmer grip on power.

At first, the notion of a more controlled world gained wide acceptance and approval. Citizens willingly gave up some of their sovereignty – and their privacy – to more paternalistic states in exchange for greater safety and stability. Citizens were more tolerant, and even eager, for top-down direction and oversight, and national leaders had more latitude to impose order in the ways they saw fit.

In developed countries, this heightened oversight took many forms: biometric IDs for all citizens, for example, and tighter regulation of key industries whose stability was deemed vital to national interests. In many developed countries, enforced cooperation with a suite of new regulations and agreements slowly but steadily restored both order and, importantly, economic growth.

There we have the prophetic Rockefellers in 2010 and three years later came their paper for the Global Health Summit in Beijing, China, when government representatives, the private sector, international organisations and groups met to discuss the next 100 years of 'global health'. The Rockefeller Foundation-funded paper was called 'Dreaming the Future of Health for the Next 100 Years and more prophecy ensued as it described a dystopian future: 'The abundance of data, digitally tracking and linking people may mean the 'death of privacy' and may replace physical interaction with transient, virtual connection, generating isolation and raising questions of how values are shaped in virtual networks.' Next in the 'Covid' hoax preparation sequence came a 'table top' simulation in 2018 for another 'imaginary' pandemic of a disease called Clade X which was said to kill 900 million people. The exercise was organised by the Gates-funded Johns Hopkins University's Center for Health Security in the United States and this is the very same university that has been compiling the disgustingly and systematically erroneous global figures for 'Covid' cases and deaths. Similar Johns Hopkins health crisis scenarios have included the Dark Winter exercise in 2001 and Atlantic Storm in 2005.

Nostradamus 201

For sheer predictive genius look no further prophecy-watchers than the Bill Gates-funded Event 201 held only six weeks before the 'coronavirus pandemic' is supposed to have broken out in China and Event 201 was based on a scenario of a global 'coronavirus pandemic'. Melinda Gates, the great man's missus, told the BBC that he had 'prepared for years' for a coronavirus pandemic which told us what we already knew. Nostradamugates had predicted in a TED talk in 2015 that a pandemic was coming that would kill a lot of people and demolish the world economy. My god, the man is a machine – possibly even literally. Now here he was only weeks before the real thing funding just such a simulated scenario and involving his friends and associates at Johns Hopkins, the World Economic Forum Cult-front of Klaus Schwab, the United Nations,

Johnson & Johnson, major banks, and officials from China and the Centers for Disease Control in the United States. What synchronicity – Johns Hopkins would go on to compile the fraudulent 'Covid' figures, the World Economic Forum and Schwab would push the 'Great Reset' in response to 'Covid', the Centers for Disease Control would be at the forefront of 'Covid' policy in the United States, Johnson & Johnson would produce a 'Covid vaccine', and everything would officially start just weeks later in China. Spooky, eh? They were even accurate in creating a simulation of a 'virus' pandemic because the 'real thing' would also be a simulation. Event 201 was not an exercise preparing for something that might happen; it was a rehearsal for what those in control knew was *going* to happen and very shortly. Hours of this simulation were posted on the Internet and the various themes and responses mirrored what would soon be imposed to transform human society. News stories were inserted and what they said would be commonplace a few weeks later with still more prophecy perfection. Much discussion focused on the need to deal with misinformation and the 'anti-vax movement' which is exactly what happened when the 'virus' arrived – was said to have arrived – in the West.

Cult-owned social media banned criticism and exposure of the official 'virus' narrative and when I said there *was* no 'virus' in early April, 2020, I was banned by one platform after another including YouTube, Facebook and later Twitter. The mainstream broadcast media in Britain was in effect banned from interviewing me by the Tony-Blair-created government broadcasting censor Ofcom headed by career government bureaucrat Melanie Dawes who was appointed just as the 'virus' hoax was about to play out in January, 2020. At the same time the Ickonic media platform was using Vimeo, another ultra-Zionist-owned operation, while our own player was being created and they deleted in an instant hundreds of videos, documentaries, series and shows to confirm their unbelievable vindictiveness. We had copies, of course, and they had to be restored one by one when our player was ready. These people have no class. Sabbatian Facebook promised free advertisements for the Gates-

controlled World Health Organization narrative while deleting 'false claims and conspiracy theories' to stop 'misinformation' about the alleged coronavirus. All these responses could be seen just a short while earlier in the scenarios of Event 201. Extreme censorship was absolutely crucial for the Cult because the official story was so ridiculous and unsupportable by the evidence that it could never survive open debate and the free-flow of information and opinion. If you can't win a debate then don't have one is the Cult's approach throughout history. Facebook's little boy front man – front boy – Mark Zuckerberg equated 'credible and accurate information' with official sources and exposing their lies with 'misinformation'.

Silencing those that can see

The censorship dynamic of Event 201 is now the norm with an army of narrative-supporting 'fact-checker' organisations whose entire reason for being is to tell the public that official narratives are true and those exposing them are lying. One of the most appalling of these 'fact-checkers' is called NewsGuard founded by ultra-Zionist Americans Gordon Crovitz and Steven Brill. Crovitz is a former publisher of *The Wall Street Journal*, former Executive Vice President of Dow Jones, a member of the Council on Foreign Relations (CFR), and on the board of the American Association of Rhodes Scholars. The CFR and Rhodes Scholarships, named after Rothschild agent Cecil Rhodes who plundered the gold and diamonds of South Africa for his masters and the Cult, have featured widely in my books. NewsGuard don't seem to like me for some reason – I really can't think why – and they have done all they can to have me censored and discredited which is, to quote an old British politician, like being savaged by a dead sheep. They are, however, like all in the censorship network, very well connected and funded by organisations themselves funded by, or connected to, Bill Gates. As you would expect with anything associated with Gates NewsGuard has an offshoot called HealthGuard which 'fights online health care hoaxes'. How very kind. Somehow the NewsGuard European Managing Director Anna-Sophie Harling, a remarkably young-

looking woman with no broadcasting experience and little hands-on work in journalism, has somehow secured a position on the 'Content Board' of UK government broadcast censor Ofcom. An executive of an organisation seeking to discredit dissidents of the official narratives is making decisions for the government broadcast 'regulator' about content?? Another appalling 'fact-checker' is Full Fact funded by George Soros and global censors Google and Facebook.

It's amazing how many activists in the 'fact-checking', 'anti-hate', arena turn up in government-related positions – people like UK Labour Party activist Imran Ahmed who heads the Center for Countering Digital Hate founded by people like Morgan McSweeney, now chief of staff to the Labour Party's hapless and useless 'leader' Keir Starmer. Digital Hate – which is what it really is – uses the American spelling of Center to betray its connection to a transatlantic network of similar organisations which in 2020 shapeshifted from attacking people for 'hate' to attacking them for questioning the 'Covid' hoax and the dangers of the 'Covid vaccine'. It's just a coincidence, you understand. This is one of Imran Ahmed's hysterical statements: 'I would go beyond calling anti-vaxxers conspiracy theorists to say they are an extremist group that pose a national security risk.' No one could ever accuse this prat of understatement and he's including in that those parents who are now against vaccines after their children were damaged for life or killed by them. He's such a nice man. Ahmed does the rounds of the Woke media getting soft-ball questions from spineless 'journalists' who never ask what right he has to campaign to destroy the freedom of speech of others while he demands it for himself. There also seems to be an overrepresentation in Ofcom of people connected to the narrative-worshipping BBC. This incredible global network of narrative-support was super-vital when the 'Covid' hoax was played in the light of the mega-whopper lies that have to be defended from the spotlight cast by the most basic intelligence.

Setting the scene

The Cult plays the long game and proceeds step-by-step ensuring that everything is in place before major cards are played and they don't come any bigger than the 'Covid' hoax. The psychopaths can't handle events where the outcome isn't certain and as little as possible – preferably nothing – is left to chance. Politicians, government and medical officials who would follow direction were brought to illusory power in advance by the Cult web whether on the national stage or others like state governors and mayors of America. For decades the dynamic between officialdom, law enforcement and the public was changed from one of service to one of control and dictatorship. Behaviour manipulation networks established within government were waiting to impose the coming 'Covid' rules and regulations specifically designed to subdue and rewire the psyche of the people in the guise of protecting health. These included in the UK the Behavioural Insights Team part-owned by the British government Cabinet Office; the Scientific Pandemic Insights Group on Behaviours (SPI-B); and a whole web of intelligence and military groups seeking to direct the conversation on social media and control the narrative. Among them are the cyberwarfare (on the people) 77th Brigade of the British military which is also coordinated through the Cabinet Office as civilian and military leadership continues to combine in what they call the Fusion Doctrine. The 77th Brigade is a British equivalent of the infamous Israeli (Sabbatian) military cyberwarfare and Internet manipulation operation Unit 8200 which I expose at length in *The Trigger*. Also carefully in place were the medical and science advisers to government – many on the payroll past or present of Bill Gates – and a whole alternative structure of unelected government stood by to take control when elected parliaments were effectively closed down once the 'Covid' card was slammed on the table. The structure I have described here and so much more was installed in every major country through the Cult networks. The top-down control hierarchy looks like this: The Cult – Cult-owned Gates – the World Health Organization and Tedros – Gates-funded or controlled chief medical officers and science 'advisers' (dictators) in each country –

political 'leaders'– law enforcement – The People. Through this simple global communication and enforcement structure the policy of the Cult could be imposed on virtually the entire human population so long as they acquiesced to the fascism. With everything in place it was time for the button to be pressed in late 2019/early 2020.

These were the prime goals the Cult had to secure for its will to prevail:

1) Locking down economies, closing all but designated 'essential' businesses (Cult-owned corporations were 'essential'), and putting the population under house arrest was an imperative to destroy independent income and employment and ensure dependency on the Cult-controlled state in the Hunger Games Society. Lockdowns had to be established as the global blueprint from the start to respond to the 'virus' and followed by pretty much the entire world.

2) The global population had to be terrified into believing in a deadly 'virus' that didn't actually exist so they would unquestioningly obey authority in the belief that authority must know how best to protect them and their families. Software salesman Gates would suddenly morph into the world's health expert and be promoted as such by the Cult-owned media.

3) A method of testing that wasn't testing for the 'virus', but was only claimed to be, had to be in place to provide the illusion of 'cases' and subsequent 'deaths' that had a very different cause to the 'Covid-19' that would be scribbled on the death certificate.

4) Because there was no 'virus' and the great majority testing positive with a test not testing for the 'virus' would have no symptoms of anything the lie had to be sold that people without symptoms (without the 'virus') could still pass it on to others. This was crucial to justify for the first time quarantining – house arresting – healthy people. Without this the economy-destroying lockdown of *everybody* could not have been credibly sold.

5) The 'saviour' had to be seen as a vaccine which beyond evil drug companies were working like angels of mercy to develop as quickly as possible, with all corners cut, to save the day. The public must absolutely not know that the 'vaccine' had nothing to do with a 'virus' or that the contents were ready and waiting with a very different motive long before the 'Covid' card was even lifted from the pack.

I said in March, 2020, that the 'vaccine' would have been created way ahead of the 'Covid' hoax which justified its use and the following December an article in the New York *Intelligencer* magazine said the Moderna 'vaccine' had been 'designed' by

January, 2020. This was 'before China had even acknowledged that the disease could be transmitted from human to human, more than a week before the first confirmed coronavirus case in the United States'. The article said that by the time the first American death was announced a month later 'the vaccine had already been manufactured and shipped to the National Institutes of Health for the beginning of its Phase I clinical trial'. The 'vaccine' was actually 'designed' long before that although even with this timescale you would expect the article to ask how on earth it could have been done that quickly. Instead it asked why the 'vaccine' had not been rolled out then and not months later. Journalism in the mainstream is truly dead. I am going to detail in the next chapter why the 'virus' has never existed and how a hoax on that scale was possible, but first the foundation on which the Big Lie of 'Covid' was built.

The test that doesn't test

Fraudulent 'testing' is the bottom line of the whole 'Covid' hoax and was the means by which a 'virus' that did not exist *appeared* to exist. They could only achieve this magic trick by using a test not testing for the 'virus'. To use a test that *was* testing for the 'virus' would mean that every test would come back negative given there was no 'virus'. They chose to exploit something called the RT-PCR test invented by American biochemist Kary Mullis in the 1980s who said publicly that his PCR test … *cannot detect infectious disease*. Yes, the 'test' used worldwide to detect infectious 'Covid' to produce all the illusory 'cases' and 'deaths' compiled by Johns Hopkins and others *cannot detect infectious disease*. This fact came from the mouth of the man who invented PCR and was awarded the Nobel Prize in Chemistry in 1993 for doing so. Sadly, and incredibly conveniently for the Cult, Mullis died in August, 2019, at the age of 74 just before his test would be fraudulently used to unleash fascism on the world. He was said to have died from pneumonia which was an irony in itself. A few months later he would have had 'Covid-19' on his death certificate. I say the timing of his death was convenient because had he lived Mullis, a brilliant, honest and decent man, would have been

vociferously speaking out against the use of his test to detect 'Covid' when it was never designed, or able, to do that. I know that to be true given that Mullis made the same point when his test was used to 'detect' – not detect – HIV. He had been seriously critical of the Gallo/Montagnier claim to have isolated the HIV 'virus' and shown it to cause AIDS for which Mullis said there was no evidence. AIDS is actually not a disease but a series of diseases from which people die all the time. When they die from those *same diseases* after a positive 'test' for HIV then AIDS goes on their death certificate. I think I've heard that before somewhere. Countries instigated a policy with 'Covid' that anyone who tested positive with a test not testing for the 'virus' and died of any other cause within 28 days and even longer 'Covid-19' had to go on the death certificate. Cases have come from the test that can't test for infectious disease and the deaths are those who have died of *anything* after testing positive with a test not testing for the 'virus'. I'll have much more later about the death certificate scandal.

Mullis was deeply dismissive of the now US 'Covid' star Anthony Fauci who he said was a liar who didn't know anything about anything – 'and I would say that to his face – nothing.' He said of Fauci: 'The man thinks he can take a blood sample, put it in an electron microscope and if it's got a virus in there you'll know it – he doesn't understand electron microscopy and he doesn't understand medicine and shouldn't be in a position like he's in.' That position, terrifyingly, has made him the decider of 'Covid' fascism policy on behalf of the Cult in his role as director since 1984 of the National Institute of Allergy and Infectious Diseases (NIAID) while his record of being wrong is laughable; but being wrong, so long as it's the *right kind* of wrong, is why the Cult loves him. He'll say anything the Cult tells him to say. Fauci was made Chief Medical Adviser to the President immediately Biden took office. Biden was installed in the White House by Cult manipulation and one of his first decisions was to elevate Fauci to a position of even more control. This is a coincidence? Yes, and I identify as a flamenco dancer called Lola. How does such an incompetent criminal like Fauci remain in that

pivotal position in American health since *the 1980s*? When you serve the Cult it looks after you until you are surplus to requirements. Kary Mullis said prophetically of Fauci and his like: 'Those guys have an agenda and it's not an agenda we would like them to have … they make their own rules, they change them when they want to, and Tony Fauci does not mind going on television in front of the people who pay his salary and lie directly into the camera.' Fauci has done that almost daily since the 'Covid' hoax began. Lying is in Fauci's DNA. To make the situation crystal clear about the PCR test this is a direct quote from its inventor Kary Mullis:

> It [the PCR test] doesn't tell you that you're sick and doesn't tell you that the thing you ended up with was really going to hurt you ...'

Ask yourself why governments and medical systems the world over have been using this very test to decide who is 'infected' with the SARS-CoV-2 'virus' and the alleged disease it allegedly causes, 'Covid-19'. The answer to that question will tell you what has been going on. By the way, here's a little show-stopper – the 'new' SARS-CoV-2 'virus' was 'identified' as such right from the start using … *the PCR test not testing for the 'virus'*. If you are new to this and find that shocking then stick around. I have hardly started yet. Even worse, other 'tests', like the 'Lateral Flow Device' (LFD), are considered so useless that they have to be *confirmed* by the PCR test! Leaked emails written by Ben Dyson, adviser to UK 'Health' Secretary Matt Hancock, said they were 'dangerously unreliable'. Dyson, executive director of strategy at the Department of Health, wrote: 'As of today, someone who gets a positive LFD result in (say) London has at best a 25 per cent chance of it being a true positive, but if it is a self-reported test potentially as low as 10 per cent (on an optimistic assumption about specificity) or as low as 2 per cent (on a more pessimistic assumption).' These are the 'tests' that schoolchildren and the public are being urged to have twice a week or more and have to isolate if they get a positive. Each fake positive goes in the statistics as a 'case' no matter how ludicrously inaccurate and the

'cases' drive lockdown, masks and the pressure to 'vaccinate'. The government said in response to the email leak that the 'tests' were accurate which confirmed yet again what shocking bloody liars they are. The real false positive rate is *100 percent* as we'll see. In another 'you couldn't make it up' the UK government agreed to pay £2.8 billion to California's Innova Medical Group to supply the irrelevant lateral flow tests. The company's primary test-making centre is in China. Innova Medical Group, established in March, 2020, is owned by Pasaca Capital Inc, chaired by Chinese-American millionaire Charles Huang who was born in Wuhan.

How it works – and how it doesn't

The RT-PCR test, known by its full title of Polymerase chain reaction, is used across the world to make millions, even billions, of copies of a DNA/RNA genetic information sample. The process is called 'amplification' and means that a tiny sample of genetic material is amplified to bring out the detailed content. I stress that it is not testing for an infectious disease. It is simply amplifying a sample of genetic material. In the words of Kary Mullis: 'PCR is … just a process that's used to make a whole lot of something out of something.' To emphasise the point companies that make the PCR tests circulated around the world to 'test' for 'Covid' warn on the box that it can't be used to detect 'Covid' or infectious disease and is for research purposes only. It's okay, rest for a minute and you'll be fine. This is the test that produces the 'cases' and 'deaths' that have been used to destroy human society. All those global and national medical and scientific 'experts' demanding this destruction to 'save us' *KNOW* that the test is not testing for the 'virus' and the cases and deaths they claim to be real are an almost unimaginable fraud. Every one of them and so many others including politicians and psychopaths like Gates and Tedros must be brought before Nuremburg-type trials and jailed for the rest of their lives. The more the genetic sample is amplified by PCR the more elements of that material become sensitive to the test and by that I don't mean sensitive for a 'virus' but for elements of the genetic material which

is *naturally* in the body or relates to remnants of old conditions of various kinds lying dormant and causing no disease. Once the amplification of the PCR reaches a certain level *everyone* will test positive. So much of the material has been made sensitive to the test that everyone will have some part of it in their body. Even lying criminals like Fauci have said that once PCR amplifications pass 35 cycles everything will be a false positive that cannot be trusted for the reasons I have described. I say, like many proper doctors and scientists, that 100 percent of the 'positives' are false, but let's just go with Fauci for a moment.

He says that any amplification over 35 cycles will produce false positives and yet the US Centers for Disease Control (CDC) and Food and Drug Administration (FDA) have recommended up to *40 cycles* and the National Health Service (NHS) in Britain admitted in an internal document for staff that it was using *45 cycles* of amplification. A long list of other countries has been doing the same and at least one 'testing' laboratory has been using *50 cycles*. Have you ever heard a doctor, medical 'expert' or the media ask what level of amplification has been used to claim a 'positive'. The 'test' comes back 'positive' and so you have the 'virus', end of story. Now we can see how the government in Tanzania could send off samples from a goat and a pawpaw fruit under human names and both came back positive for 'Covid-19'. Tanzania president John Magufuli mocked the 'Covid' hysteria, the PCR test and masks and refused to import the DNA-manipulating 'vaccine'. The Cult hated him and an article sponsored by the Bill Gates Foundation appeared in the London *Guardian* in February, 2021, headed 'It's time for Africa to rein in Tanzania's anti-vaxxer president'. Well, 'reined in' he shortly was. Magufuli appeared in good health, but then, in March, 2021, he was dead at 61 from 'heart failure'. He was replaced by Samia Hassan Suhulu who is connected to Klaus Schwab's World Economic Forum and she immediately reversed Magufuli's 'Covid' policy. A sample of cola tested positive for 'Covid' with the PCR test in Germany while American actress and singer-songwriter Erykah Badu tested positive in one nostril and negative in the other. Footballer Ronaldo called

the PCR test 'bullshit' after testing positive three times and being forced to quarantine and miss matches when there was nothing wrong with him. The mantra from Tedros at the World Health Organization and national governments (same thing) has been test, test, test. They know that the more tests they can generate the more fake 'cases' they have which go on to become 'deaths' in ways I am coming to. The UK government has its Operation Moonshot planned to test multiple millions every day in workplaces and schools with free tests for everyone to use twice a week at home in line with the Cult plan from the start to make testing part of life. A government advertisement for an 'Interim Head of Asymptomatic Testing Communication' said the job included responsibility for delivering a 'communications strategy' (propaganda) 'to support the expansion of asymptomatic testing that *'normalises testing as part of everyday life*'. More tests means more fake 'cases', 'deaths' and fascism. I have heard of, and from, many people who booked a test, couldn't turn up, and yet got a positive result through the post for a test they'd never even had. The whole thing is crazy, but for the Cult there's method in the madness. Controlling and manipulating the level of amplification of the test means the authorities can control whenever they want the number of apparent 'cases' and 'deaths'. If they want to justify more fascist lockdown and destruction of livelihoods they keep the amplification high. If they want to give the illusion that lockdowns and the 'vaccine' are working then they lower the amplification and 'cases' and 'deaths' will appear to fall. In January, 2021, the Cult-owned World Health Organization suddenly warned laboratories about over-amplification of the test and to lower the threshold. Suddenly headlines began appearing such as: 'Why ARE "Covid" cases plummeting?' This was just when the vaccine rollout was underway and I had predicted months before they would make cases appear to fall through amplification tampering when the 'vaccine' came. These people are so predictable.

Cow vaccines?

The question must be asked of what is on the test swabs being poked far up the nose of the population to the base of the brain? A nasal swab punctured one woman's brain and caused it to leak fluid. Most of these procedures are being done by people with little training or medical knowledge. Dr Lorraine Day, former orthopaedic trauma surgeon and Chief of Orthopaedic Surgery at San Francisco General Hospital, says the tests are really a *'vaccine'*. Cows have long been vaccinated this way. She points out that masks have to cover the nose and the mouth where it is claimed the 'virus' exists in saliva. Why then don't they take saliva from the mouth as they do with a DNA test instead of pushing a long swab up the nose towards the brain? The ethmoid bone separates the nasal cavity from the brain and within that bone is the cribriform plate. Dr Day says that when the swab is pushed up against this plate and twisted the procedure is 'depositing things back there'. She claims that among these 'things' are nanoparticles that can enter the brain. Researchers have noted that a team at the Gates-funded Johns Hopkins have designed tiny, star-shaped micro-devices that can latch onto intestinal mucosa and release drugs into the body. Mucosa is the thin skin that covers the inside surface of parts of the body such as *the nose* and mouth and produces mucus to protect them. The Johns Hopkins micro-devices are called 'theragrippers' and were 'inspired' by a parasitic worm that digs its sharp teeth into a host's intestines. Nasal swabs are also coated in the sterilisation agent ethylene oxide. The US National Cancer Institute posts this explanation on its website:

> At room temperature, ethylene oxide is a flammable colorless gas with a sweet odor. It is used primarily to produce other chemicals, including antifreeze. In smaller amounts, ethylene oxide is used as a pesticide and a sterilizing agent. The ability of ethylene oxide to damage DNA makes it an effective sterilizing agent but also accounts for its cancer-causing activity.

The Institute mentions lymphoma and leukaemia as cancers most frequently reported to be associated with occupational exposure to ethylene oxide along with stomach and breast cancers. How does anyone think this is going to work out with the constant testing

regime being inflicted on adults and children at home and at school that will accumulate in the body anything that's on the swab?

Doctors know best

It is vital for people to realise that 'hero' doctors 'know' only what the Big Pharma-dominated medical authorities tell them to 'know' and if they refuse to 'know' what they are told to 'know' they are out the door. They are mostly not physicians or healers, but repeaters of the official narrative – or else. I have seen alleged professional doctors on British television make shocking statements that we are supposed to take seriously. One called 'Dr' Amir Khan, who is actually telling patients how to respond to illness, said that men could take the birth pill to 'help slow down the effects of Covid-19'. In March, 2021, another ridiculous 'Covid study' by an American doctor proposed injecting men with the female sex hormone progesterone as a 'Covid' treatment. British doctor Nighat Arif told the BBC that face coverings were now going to be part of ongoing normal. Yes, the vaccine protects you, she said (evidence?) … but the way to deal with viruses in the community was always going to come down to hand washing, face covering and keeping a physical distance. That's not what we were told before the 'vaccine' was circulating. Arif said she couldn't imagine ever again going on the underground or in a lift without a mask. I was just thanking my good luck that she was not my doctor when she said – in March, 2021 – that if 'we are *behaving* and we are doing all the right things' she thought we could 'have our nearest and dearest around us at home … around *Christmas* and N*ew Year!* Her patronising delivery was the usual school teacher talking to six-year-olds as she repeated every government talking point and probably believed them all. If we have learned anything from the 'Covid' experience surely it must be that humanity's perception of doctors needs a fundamental rethink. NHS 'doctor' Sara Kayat told her television audience that the 'Covid vaccine' would '100 percent prevent hospitalisation and death'. Not even Big Pharma claimed that. We have to stop taking 'experts' at their word without question when so many of them are

clueless and only repeating the party line on which their careers depend. That is not to say there are not brilliants doctors – there are and I have spoken to many of them since all this began – but you won't see them in the mainstream media or quoted by the psychopaths and yes-people in government.

Remember the name – Christian Drosten

German virologist Christian Drosten, Director of Charité Institute of Virology in Berlin, became a national star after the pandemic hoax began. He was feted on television and advised the German government on 'Covid' policy. Most importantly to the wider world Drosten led a group that produced the 'Covid' testing protocol for the PCR test. What a remarkable feat given the PCR cannot test for infectious disease and even more so when you think that Drosten said that his method of testing for SARS-CoV-2 was developed 'without having virus material available'. *He developed a test for a 'virus' that he didn't have and had never seen*. Let that sink in as you survey the global devastation that came from what he did. The whole catastrophe of Drosten's 'test' was based on the alleged genetic sequence published by Chinese scientists on the Internet. We will see in the next chapter that this alleged 'genetic sequence' has never been produced by China or anyone and cannot be when there *is no* SARS-CoV-2. Drosten, however, doesn't seem to let little details like that get in the way. He was the lead author with Victor Corman from the same Charité Hospital of the paper 'Detection of 2019 novel coronavirus (2019-nCoV) by real-time PCR' published in a magazine called *Eurosurveillance*. This became known as the Corman-Drosten paper. In November, 2020, with human society devastated by the effects of the Corman-Drosten test baloney, the protocol was publicly challenged by 22 international scientists and independent researchers from Europe, the United States, and Japan. Among them were senior molecular geneticists, biochemists, immunologists, and microbiologists. They produced a document headed 'External peer review of the RTPCR test to detect SARS-Cov-2 Reveals 10 Major Flaws At The Molecular and Methodological Level: Consequences

For False-Positive Results'. The flaws in the Corman-Drosten test included the following:

- The test is non-specific because of erroneous design
- Results are enormously variable
- The test is unable to discriminate between the whole 'virus' and viral fragments
- It doesn't have positive or negative controls
- The test lacks a standard operating procedure
- It is unsupported by proper peer view

The scientists said the PCR 'Covid' testing protocol was not founded on science and they demanded the Corman-Drosten paper be retracted by *Eurosurveillance*. They said all present and previous Covid deaths, cases, and 'infection rates' should be subject to a massive retroactive inquiry. Lockdowns and travel restrictions should be reviewed and relaxed and those diagnosed through PCR to have 'Covid-19' should not be forced to isolate. Dr Kevin Corbett, a health researcher and nurse educator with a long academic career producing a stream of peer-reviewed publications at many UK universities, made the same point about the PCR test debacle. He said of the scientists' conclusions: 'Every scientific rationale for the development of that test has been totally destroyed by this paper. It's like Hiroshima/Nagasaki to the Covid test.' He said that China hadn't given them an isolated 'virus' when Drosten developed the test. Instead they had developed the test from *a sequence in a gene bank*.' Put another way … *they made it up*! The scientists were supported in this contention by a Portuguese appeals court which ruled in November, 2020, that PCR tests are unreliable and it is unlawful to quarantine people based solely on a PCR test. The point about China not providing an isolated virus must be true when the 'virus' has never been isolated to this day and the consequences of that will become clear. Drosten and company produced this useless 'protocol' right on cue in January, 2020, just as the 'virus' was said to

be moving westward and it somehow managed to successfully pass a peer-review in 24 hours. In other words there was no peer-review for a test that would be used to decide who had 'Covid' and who didn't across the world. The Cult-created, Gates-controlled World Health Organization immediately recommended all its nearly 200 member countries to use the Drosten PCR protocol to detect 'cases' and 'deaths'. The sting was underway and it continues to this day.

So who is this Christian Drosten that produced the means through which death, destruction and economic catastrophe would be justified? His education background, including his doctoral thesis, would appear to be somewhat shrouded in mystery and his track record is dire as with another essential player in the 'Covid' hoax, the Gates-funded Professor Neil Ferguson at the Gates-funded Imperial College in London of whom more shortly. Drosten predicted in 2003 that the alleged original SARS 'virus' (SARS-1') was an epidemic that could have serious effects on economies and an effective vaccine would take at least two years to produce. Drosten's answer to every alleged 'outbreak' is a vaccine which you won't be shocked to know. What followed were just 774 official deaths worldwide and none in Germany where there were only nine cases. That is even if you believe there ever was a SARS 'virus' when the evidence is zilch and I will expand on this in the next chapter. Drosten claims to be co-discoverer of 'SARS-1' and developed a test for it in 2003. He was screaming warnings about 'swine flu' in 2009 and how it was a widespread infection far more severe than any dangers from a vaccine could be and people should get vaccinated. It would be helpful for Drosten's vocal chords if he simply recorded the words 'the virus is deadly and you need to get vaccinated' and copies could be handed out whenever the latest made-up threat comes along. Drosten's swine flu epidemic never happened, but Big Pharma didn't mind with governments spending hundreds of millions on vaccines that hardly anyone bothered to use and many who did wished they hadn't. A study in 2010 revealed that the risk of dying from swine flu, or H1N1, was no higher than that of the annual seasonal flu which is what at least most of 'it' really was as in

the case of 'Covid-19'. A media investigation into Drosten asked how with such a record of inaccuracy he could be *the* government adviser on these issues. The answer to that question is the same with Drosten, Ferguson and Fauci – they keep on giving the authorities the 'conclusions' and 'advice' they want to hear. Drosten certainly produced the goods for them in January, 2020, with his PCR protocol garbage and provided the foundation of what German internal medicine specialist Dr Claus Köhnlein, co-author of *Virus Mania*, called the 'test pandemic'. The 22 scientists in the *Eurosurveillance* challenge called out conflicts of interest within the Drosten 'protocol' group and with good reason. Olfert Landt, a regular co-author of Drosten 'studies', owns the biotech company TIB Molbiol Syntheselabor GmbH in Berlin which manufactures and sells the tests that Drosten and his mates come up with. They have done this with SARS, Enterotoxigenic E. coli (ETEC), MERS, Zika 'virus', yellow fever, and now 'Covid'. Landt told the *Berliner Zeitung* newspaper:

> The testing, design and development came from the Charité [Drosten and Corman]. We simply implemented it immediately in the form of a kit. And if we don't have the virus, which originally only existed in Wuhan, we can make a synthetic gene to simulate the genome of the virus. That's what we did very quickly.

This is more confirmation that the Drosten test was designed without access to the 'virus' and only a synthetic simulation which is what SARS-CoV-2 really is – a computer-generated synthetic fiction. It's quite an enterprise they have going here. A Drosten team decides what the test for something should be and Landt's biotech company flogs it to governments and medical systems across the world. His company must have made an absolute fortune since the 'Covid' hoax began. Dr Reiner Fuellmich, a prominent German consumer protection trial lawyer in Germany and California, is on Drosten's case and that of Tedros at the World Health Organization for crimes against humanity with a class-action lawsuit being prepared in the United States and other legal action in Germany.

Why China?

Scamming the world with a 'virus' that doesn't exist would seem impossible on the face of it, but not if you have control of the relatively few people that make policy decisions and the great majority of the global media. Remember it's not about changing 'real' reality it's about controlling *perception* of reality. You don't have to make something happen you only have make people *believe* that it's happening. Renegade Minds understand this and are therefore much harder to swindle. 'Covid-19' is not a 'real' 'virus'. It's a mind virus, like a computer virus, which has infected the minds, not the bodies, of billions. It all started, publically at least, in China and that alone is of central significance. The Cult was behind the revolution led by its asset Mao Zedong, or Chairman Mao, which established the People's Republic of China on October 1st, 1949. It should have been called The Cult's Republic of China, but the name had to reflect the recurring illusion that vicious dictatorships are run by and for the people (see all the 'Democratic Republics' controlled by tyrants). In the same way we have the 'Biden' Democratic Republic of America officially ruled by a puppet tyrant (at least temporarily) on behalf of Cult tyrants. The creation of Mao's merciless communist/fascist dictatorship was part of a frenzy of activity by the Cult at the conclusion of World War Two which, like the First World War, it had instigated through its assets in Germany, Britain, France, the United States and elsewhere. Israel was formed in 1948; the Soviet Union expanded its 'Iron Curtain' control, influence and military power with the Warsaw Pact communist alliance in 1955; the United Nations was formed in 1945 as a Cult precursor to world government; and a long list of world bodies would be established including the World Health Organization (1948), World Trade Organization (1948 under another name until 1995), International Monetary Fund (1945) and World Bank (1944). Human society was redrawn and hugely centralised in the global Problem-Reaction-Solution that was World War Two. All these changes were significant. Israel would become the headquarters of the Sabbatians

and the revolution in China would prepare the ground and control system for the events of 2019/2020.

Renegade Minds know there are no borders except for public consumption. The Cult is a seamless, borderless global entity and to understand the game we need to put aside labels like borders, nations, countries, communism, fascism and democracy. These delude the population into believing that countries are ruled within their borders by a government of whatever shade when these are mere agencies of a global power. America's illusion of democracy and China's communism/fascism are subsidiaries – vehicles – for the same agenda. We may hear about conflict and competition between America and China and on the lower levels that will be true; but at the Cult level they are branches of the same company in the way of the McDonald's example I gave earlier. I have tracked in the books over the years support by US governments of both parties for Chinese Communist Party infiltration of American society through allowing the sale of land, even military facilities, and the acquisition of American business and university influence. All this is underpinned by the infamous stealing of intellectual property and technological know-how. Cult-owned Silicon Valley corporations waive their fraudulent 'morality' to do business with human-rights-free China; Cult-controlled Disney has become China's PR department; and China in effect owns 'American' sports such as basketball which depends for much of its income on Chinese audiences. As a result any sports player, coach or official speaking out against China's horrific human rights record is immediately condemned or fired by the China-worshipping National Basketball Association. One of the first acts of China-controlled Biden was to issue an executive order telling federal agencies to stop making references to the 'virus' by the 'geographic location of its origin'. Long-time Congressman Jerry Nadler warned that criticising China, America's biggest rival, leads to hate crimes against Asian people in the United States. So shut up you bigot. China is fast closing in on Israel as a country that must not be criticised which is apt, really, given that Sabbatians control them both. The two countries have

developed close economic, military, technological and strategic ties which include involvement in China's 'Silk Road' transport and economic initiative to connect China with Europe. Israel was the first country in the Middle East to recognise the establishment of Mao's tyranny in 1950 months after it was established.

Project Wuhan – the 'Covid' Psyop

I emphasise again that the Cult plays the long game and what is happening to the world today is the result of centuries of calculated manipulation following a script to take control step-by-step of every aspect of human society. I will discuss later the common force behind all this that has spanned those centuries and thousands of years if the truth be told. Instigating the Mao revolution in China in 1949 with a 2020 'pandemic' in mind is not only how they work – the 71 years between them is really quite short by the Cult's standards of manipulation preparation. The reason for the Cult's Chinese revolution was to create a fiercely-controlled environment within which an extreme structure for human control could be incubated to eventually be unleashed across the world. We have seen this happen since the 'pandemic' emerged from China with the Chinese control-structure founded on AI technology and tyrannical enforcement sweep across the West. Until the moment when the Cult went for broke in the West and put its fascism on public display Western governments had to pay some lip-service to freedom and democracy to not alert too many people to the tyranny-in-the-making. Freedoms were more subtly eroded and power centralised with covert government structures put in place waiting for the arrival of 2020 when that smokescreen of 'freedom' could be dispensed with. The West was not able to move towards tyranny before 2020 anything like as fast as China which was created as a tyranny and had no limits on how fast it could construct the Cult's blueprint for global control. When the time came to impose that structure on the world it was the same Cult-owned Chinese communist/fascist government that provided the excuse – the 'Covid pandemic'. It was absolutely crucial to the Cult plan for the Chinese response to the 'pandemic' –

draconian lockdowns of the entire population – to become the blueprint that Western countries would follow to destroy the livelihoods and freedom of their people. This is why the Cult-owned, Gates-owned, WHO Director-General Tedros said early on:

The Chinese government is to be congratulated for the extraordinary measures it has taken to contain the outbreak. China is actually setting a new standard for outbreak response and it is not an exaggeration.

Forbes magazine said of China: '… those measures protected untold millions from getting the disease'. The Rockefeller Foundation 'epidemic scenario' document in 2010 said 'prophetically':

However, a few countries did fare better – China in particular. The Chinese government's quick imposition and enforcement of mandatory quarantine for all citizens, as well as its instant and near-hermetic sealing off of all borders, saved millions of lives, stopping the spread of the virus far earlier than in other countries and enabling a swifter post-pandemic recovery.

Once again – *spooky*.

The first official story was the 'bat theory' or rather the bat diversion. The source of the 'virus outbreak' we were told was a ''wet market' in Wuhan where bats and other animals are bought and eaten in horrifically unhygienic conditions. Then another story emerged through the alternative media that the 'virus' had been released on purpose or by accident from a BSL-4 (biosafety level 4) laboratory in Wuhan not far from the wet market. The lab was reported to create and work with lethal concoctions and bioweapons. Biosafety level 4 is the highest in the World Health Organization system of safety and containment. Renegade Minds are aware of what I call designer manipulation. The ideal for the Cult is for people to buy its prime narrative which in the opening salvoes of the 'pandemic' was the wet market story. It knows, however, that there is now a considerable worldwide alternative media of researchers sceptical of anything governments say and they are often given a version of events in a form they can perceive as credible while misdirecting them from the real truth. In this case let them

think that the conspiracy involved is a 'bioweapon virus' released from the Wuhan lab to keep them from the real conspiracy – *there is no 'virus'*. The WHO's current position on the source of the outbreak at the time of writing appears to be: 'We haven't got a clue, mate.' This is a good position to maintain mystery and bewilderment. The inner circle will know where the 'virus' came from – *nowhere*. The bottom line was to ensure the public believed there *was* a 'virus' and it didn't much matter if they thought it was natural or had been released from a lab. The belief that there was a 'deadly virus' was all that was needed to trigger global panic and fear. The population was terrified into handing their power to authority and doing what they were told. They had to or they were 'all gonna die'.

In March, 2020, information began to come my way from real doctors and scientists and my own additional research which had my intuition screaming: 'Yes, that's it! *There is no virus*.' The 'bioweapon' was not the 'virus'; it was the *'vaccine'* already being talked about that would be the bioweapon. My conclusion was further enhanced by happenings in Wuhan. The 'virus' was said to be sweeping the city and news footage circulated of people collapsing in the street (which they've never done in the West with the same 'virus'). The Chinese government was building 'new hospitals' in a matter of ten days to 'cope with demand' such was the virulent nature of the 'virus'. Yet in what seemed like no time the 'new hospitals' closed – even if they even opened – and China declared itself 'virus-free'. It was back to business as usual. This was more propaganda to promote the Chinese draconian lockdowns in the West as the way to 'beat the virus'. Trouble was that we subsequently had lockdown after lockdown, but never business as usual. As the people of the West and most of the rest of the world were caught in an ever-worsening spiral of lockdown, social distancing, masks, isolated old people, families forced apart, and livelihood destruction, it was party-time in Wuhan. Pictures emerged of thousands of people enjoying pool parties and concerts. It made no sense until you realised there never was a 'virus' and the

whole thing was a Cult set-up to transform human society out of one its major global strongholds – China.

How is it possible to deceive virtually the entire world population into believing there is a deadly virus when there is not even a 'virus' let alone a deadly one? It's nothing like as difficult as you would think and that's clearly true because it happened.

Postscript: See end of book Postscript for more on the 'Wuhan lab virus release' story which the authorities and media were pushing heavily in the summer of 2021 to divert attention from the truth that the 'Covid virus' is pure invention.

CHAPTER FIVE

There *is no* 'virus'

You can fool some of the people all of the time, and all of the people some of the time, but you cannot fool all of the people all of the time
Abraham Lincoln

The greatest form of mind control is repetition. The more you repeat the same mantra of alleged 'facts' the more will accept them to be true. It becomes an 'everyone knows that, mate'. If you can also censor any other version or alternative to your alleged 'facts' you are pretty much home and cooking.

By the start of 2020 the Cult owned the global mainstream media almost in its entirety to spew out its 'Covid' propaganda and ignore or discredit any other information and view. Cult-owned social media platforms in Cult-owned Silicon Valley were poised and ready to unleash a campaign of ferocious censorship to obliterate all but the official narrative. To complete the circle many demands for censorship by Silicon Valley were led by the mainstream media as 'journalists' became full-out enforcers for the Cult both as propagandists and censors. Part of this has been the influx of young people straight out of university who have become 'journalists' in significant positions. They have no experience and a headful of programmed perceptions from their years at school and university at a time when today's young are the most perceptually-targeted generations in known human history given the insidious impact of technology. They enter the media perceptually prepared and ready to repeat the narratives of the system that programmed them to

repeat its narratives. The BBC has a truly pathetic 'specialist disinformation reporter' called Marianna Spring who fits this bill perfectly. She is clueless about the world, how it works and what is really going on. Her role is to discredit anyone doing the job that a proper journalist would do and system-serving hacks like Spring wouldn't dare to do or even see the need to do. They are too busy licking the arse of authority which can never be wrong and, in the case of the BBC propaganda programme, *Panorama*, contacting payments systems such as PayPal to have a donations page taken down for a film company making documentaries questioning vaccines. Even the BBC soap opera *EastEnders* included a disgracefully biased scene in which an inarticulate white working class woman was made to look foolish for questioning the 'vaccine' while a well-spoken black man and Asian woman promoted the government narrative. It ticked every BBC box and the fact that the black and minority community was resisting the 'vaccine' had nothing to do with the way the scene was written. The BBC has become a disgusting tyrannical propaganda and censorship operation that should be defunded and disbanded and a free media take its place with a brief to stop censorship instead of demanding it. A BBC 'interview' with Gates goes something like: 'Mr Gates, sir, if I can call you sir, would you like to tell our audience why you are such a great man, a wonderful humanitarian philanthropist, and why you should absolutely be allowed as a software salesman to decide health policy for approaching eight billion people? Thank you, sir, please sir.' Propaganda programming has been incessant and merciless and when all you hear is the same story from the media, repeated by those around you who have only heard the same story, is it any wonder that people on a grand scale believe absolute mendacious garbage to be true? You are about to see, too, why this level of information control is necessary when the official 'Covid' narrative is so nonsensical and unsupportable by the evidence.

Structure of Deceit

The pyramid structure through which the 'Covid' hoax has been manifested is very simple and has to be to work. As few people as possible have to be involved with full knowledge of what they are doing – and why – or the real story would get out. At the top of the pyramid are the inner core of the Cult which controls Bill Gates who, in turn, controls the World Health Organization through his pivotal funding and his puppet Director-General mouthpiece, Tedros. Before he was appointed Tedros was chair of the Gates-founded Global Fund to 'fight against AIDS, tuberculosis and malaria', a board member of the Gates-funded 'vaccine alliance' GAVI, and on the board of another Gates-funded organisation. Gates owns him and picked him for a specific reason – Tedros is a crook and worse. 'Dr' Tedros (he's not a medical doctor, the first WHO chief not to be) was a member of the tyrannical Marxist government of Ethiopia for decades with all its human rights abuses. He has faced allegations of corruption and misappropriation of funds and was exposed three times for covering up cholera epidemics while Ethiopia's health minister. Tedros appointed the mass-murdering genocidal Zimbabwe dictator Robert Mugabe as a WHO goodwill ambassador for public health which, as with Tedros, is like appointing a psychopath to run a peace and love campaign. The move was so ridiculous that he had to drop Mugabe in the face of widespread condemnation. American economist David Steinman, a Nobel peace prize nominee, lodged a complaint with the International Criminal Court in The Hague over alleged genocide by Tedros when he was Ethiopia's foreign minister. Steinman says Tedros was a 'crucial decision maker' who directed the actions of Ethiopia's security forces from 2013 to 2015 and one of three officials in charge when those security services embarked on the 'killing' and 'torturing' of Ethiopians. You can see where Tedros is coming from and it's sobering to think that he has been the vehicle for Gates and the Cult to direct the global response to 'Covid'. Think about that. A psychopathic Cult dictates to psychopath Gates who dictates to psychopath Tedros who dictates how countries of the world must respond to a 'Covid virus' never scientifically shown to exist. At the same time psychopathic Cult-owned Silicon Valley information

giants like Google, YouTube, Facebook and Twitter announced very early on that they would give the Cult/Gates/Tedros/WHO version of the narrative free advertising and censor those who challenged their intelligence-insulting, mendacious story.

The next layer in the global 'medical' structure below the Cult, Gates and Tedros are the chief medical officers and science 'advisers' in each of the WHO member countries which means virtually all of them. Medical officers and arbiters of science (they're not) then take the WHO policy and recommended responses and impose them on their country's population while the political 'leaders' say they are deciding policy (they're clearly not) by 'following the science' on the advice of the 'experts' – the same medical officers and science 'advisers' (dictators). In this way with the rarest of exceptions the entire world followed the same policy of lockdown, people distancing, masks and 'vaccines' dictated by the psychopathic Cult, psychopathic Gates and psychopathic Tedros who we are supposed to believe give a damn about the health of the world population they are seeking to enslave. That, amazingly, is all there is to it in terms of crucial decision-making. Medical staff in each country then follow like sheep the dictates of the shepherds at the top of the national medical hierarchies – chief medical officers and science 'advisers' who themselves follow like sheep the shepherds of the World Health Organization and the Cult. Shepherds at the national level often have major funding and other connections to Gates and his Bill and Melinda Gates Foundation which carefully hands out money like confetti at a wedding to control the entire global medical system from the WHO down.

Follow the money

Christopher Whitty, Chief Medical Adviser to the UK Government at the centre of 'virus' policy, a senior adviser to the government's Scientific Advisory Group for Emergencies (SAGE), and Executive Board member of the World Health Organization, was gifted a grant of $40 million by the Bill and Melinda Gates Foundation for malaria research in Africa. The BBC described the unelected Whitty as 'the

official who will probably have the greatest impact on our everyday lives of any individual policymaker in modern times' and so it turned out. What Gates and Tedros have said Whitty has done like his equivalents around the world. Patrick Vallance, co-chair of SAGE and the government's Chief Scientific Adviser, is a former executive of Big Pharma giant GlaxoSmithKline with its fundamental financial and business connections to Bill Gates. In September, 2020, it was revealed that Vallance owned a deferred bonus of shares in GlaxoSmithKline worth £600,000 while the company was 'developing' a 'Covid vaccine'. Move along now – nothing to see here – what could possibly be wrong with that? Imperial College in London, a major player in 'Covid' policy in Britain and elsewhere with its 'Covid-19' Response Team, is funded by Gates and has big connections to China while the now infamous Professor Neil Ferguson, the useless 'computer modeller' at Imperial College is also funded by Gates. Ferguson delivered the dramatically inaccurate excuse for the first lockdowns (much more in the next chapter). The Institute for Health Metrics and Evaluation (IHME) in the United States, another source of outrageously false 'Covid' computer models to justify lockdowns, is bankrolled by Gates who is a vehement promotor of lockdowns. America's version of Whitty and Vallance, the again now infamous Anthony Fauci, has connections to 'Covid vaccine' maker Moderna as does Bill Gates through funding from the Bill and Melinda Gates Foundation. Fauci is director of the National Institute of Allergy and Infectious Diseases (NIAID), a major recipient of Gates money, and they are very close. Deborah Birx who was appointed White House Coronavirus Response Coordinator in February, 2020, is yet another with ties to Gates. Everywhere you look at the different elements around the world behind the coordination and decision making of the 'Covid' hoax there is Bill Gates and his money. They include the World Health Organization; Centers for Disease Control (CDC) in the United States; National Institutes of Health (NIH) of Anthony Fauci; Imperial College and Neil Ferguson; the London School of Hygiene where Chris Whitty worked; Regulatory agencies like the UK Medicines & Healthcare products Regulatory Agency (MHRA)

which gave emergency approval for 'Covid vaccines'; Wellcome Trust; GAVI, the Vaccine Alliance; the Coalition for Epidemic Preparedness Innovations (CEPI); Johns Hopkins University which has compiled the false 'Covid' figures; and the World Economic Forum. A Nationalfile.com article said:

> Gates has a lot of pull in the medical world, he has a multi-million dollar relationship with Dr. Fauci, and Fauci originally took the Gates line supporting vaccines and casting doubt on [the drug hydroxychloroquine]. Coronavirus response team member Dr. Deborah Birx, appointed by former president Obama to serve as United States Global AIDS Coordinator, also sits on the board of a group that has received billions from Gates' foundation, and Birx reportedly used a disputed Bill Gates-funded model for the White House's Coronavirus effort. Gates is a big proponent for a population lockdown scenario for the Coronavirus outbreak.

Another funder of Moderna is the Defense Advanced Research Projects Agency (DARPA), the technology-development arm of the Pentagon and one of the most sinister organisations on earth. DARPA had a major role with the CIA covert technology-funding operation In-Q-Tel in the development of Google and social media which is now at the centre of global censorship. Fauci and Gates are extremely close and openly admit to talking regularly about 'Covid' policy, but then why wouldn't Gates have a seat at every national 'Covid' table after his Foundation committed $1.75 billion to the 'fight against Covid-19'. When passed through our Orwellian Translation Unit this means that he has bought and paid for the Cult-driven 'Covid' response worldwide. Research the major 'Covid' response personnel in your own country and you will find the same Gates funding and other connections again and again. Medical and science chiefs following World Health Organization 'policy' sit atop a medical hierarchy in their country of administrators, doctors and nursing staff. These 'subordinates' are told they must work and behave in accordance with the policy delivered from the 'top' of the national 'health' pyramid which is largely the policy delivered by the WHO which is the policy delivered by Gates and the Cult. The whole 'Covid' narrative has been imposed on medical staff by a climate of fear although great numbers don't even need that to comply. They do so through breathtaking levels of ignorance and

include doctors who go through life simply repeating what Big Pharma and their hierarchical masters tell them to say and believe. No wonder Big Pharma 'medicine' is one of the biggest killers on Planet Earth.

The same top-down system of intimidation operates with regard to the Cult Big Pharma cartel which also dictates policy through national and global medical systems in this way. The Cult and Big Pharma agendas are the same because the former controls and owns the latter. 'Health' administrators, doctors, and nursing staff are told to support and parrot the dictated policy or they will face consequences which can include being fired. How sad it's been to see medical staff meekly repeating and imposing Cult policy without question and most of those who can see through the deceit are only willing to speak anonymously off the record. They know what will happen if their identity is known. This has left the courageous few to expose the lies about the 'virus', face masks, overwhelmed hospitals that aren't, and the dangers of the 'vaccine' that isn't a vaccine. When these medical professionals and scientists, some renowned in their field, have taken to the Internet to expose the truth their articles, comments and videos have been deleted by Cult-owned Facebook, Twitter and YouTube. What a real head-shaker to see YouTube videos with leading world scientists and highly qualified medical specialists with an added link underneath to the notorious Cult propaganda website *Wikipedia* to find the 'facts' about the same subject.

HIV – the 'Covid' trial-run

I'll give you an example of the consequences for health and truth that come from censorship and unquestioning belief in official narratives. The story was told by PCR inventor Kary Mullis in his book *Dancing Naked in the Mind Field*. He said that in 1984 he accepted as just another scientific fact that Luc Montagnier of France's Pasteur Institute and Robert Gallo of America's National Institutes of Health had independently discovered that a 'retrovirus' dubbed HIV (human immunodeficiency virus) caused AIDS. They

were, after all, Mullis writes, specialists in retroviruses. This is how the medical and science pyramids work. Something is announced or *assumed* and then becomes an everybody-knows-that purely through repetition of the assumption as if it is fact. Complete crap becomes accepted truth with no supporting evidence and only repetition of the crap. This is how a 'virus' that doesn't exist became the 'virus' that changed the world. The HIV-AIDS fairy story became a multi-billion pound industry and the media poured out propaganda terrifying the world about the deadly HIV 'virus' that caused the lethal AIDS. By then Mullis was working at a lab in Santa Monica, California, to detect retroviruses with his PCR test in blood donations received by the Red Cross. In doing so he asked a virologist where he could find a reference for HIV being the cause of AIDS. 'You don't need a reference,' the virologist said … *'Everybody knows it.'* Mullis said he wanted to quote a reference in the report he was doing and he said he felt a little funny about not knowing the source of such an important discovery when everyone else seemed to. The virologist suggested he cite a report by the Centers for Disease Control and Prevention (CDC) on morbidity and mortality. Mullis read the report, but it only said that an organism had been identified and did not say how. The report did not identify the original scientific work. Physicians, however, *assumed* (key recurring theme) that if the CDC was convinced that HIV caused AIDS then proof must exist. Mullis continues:

I did computer searches. Neither Montagnier, Gallo, nor anyone else had published papers describing experiments which led to the conclusion that HIV probably caused AIDS. I read the papers in Science for which they had become well known as AIDS doctors, but all they had said there was that they had found evidence of a past infection by something which was probably HIV in some AIDS patients.

They found antibodies. Antibodies to viruses had always been considered evidence of past disease, not present disease. Antibodies signaled that the virus had been defeated. The patient had saved himself. There was no indication in these papers that this virus caused a disease. They didn't show that everybody with the antibodies had the disease. In fact they found some healthy people with antibodies.

Mullis asked why their work had been published if Montagnier and Gallo hadn't really found this evidence, and why had they been fighting so hard to get credit for the discovery? He says he was hesitant to write 'HIV is the probable cause of AIDS' until he found published evidence to support that. 'Tens of thousands of scientists and researchers were spending billions of dollars a year doing research based on this idea,' Mullis writes. 'The reason had to be there somewhere; otherwise these people would not have allowed their research to settle into one narrow channel of investigation.' He said he lectured about PCR at numerous meetings where people were always talking about HIV and he asked them how they knew that HIV was the cause of AIDS:

> Everyone said something. Everyone had the answer at home, in the office, in some drawer. They all knew, and they would send me the papers as soon as they got back. But I never got any papers. Nobody ever sent me the news about how AIDS was caused by HIV.

Eventually Mullis was able to ask Montagnier himself about the reference proof when he lectured in San Diego at the grand opening of the University of California AIDS Research Center. Mullis says this was the last time he would ask his question without showing anger. Montagnier said he should reference the CDC report. 'I read it', Mullis said, and it didn't answer the question. 'If Montagnier didn't know the answer who the hell did?' Then one night Mullis was driving when an interview came on National Public Radio with Peter Duesberg, a prominent virologist at Berkeley and a California Scientist of the Year. Mullis says he finally understood why he could not find references that connected HIV to AIDS – *there weren't any!* No one had ever proved that HIV causes AIDS even though it had spawned a multi-billion pound global industry and the media was repeating this as fact every day in their articles and broadcasts terrifying the shit out of people about AIDS and giving the impression that a positive test for HIV (see 'Covid') was a death sentence. Duesberg was a threat to the AIDS gravy train and the agenda that underpinned it. He was therefore abused and castigated after he told the Proceedings of the National Academy of Sciences

there was no good evidence implicating the new 'virus'. Editors rejected his manuscripts and his research funds were deleted. Mullis points out that the CDC has defined AIDS as one of more than 30 diseases *if accompanied* by a positive result on a test that detects antibodies to HIV; but those same diseases are not defined as AIDS cases when antibodies are not detected:

> If an HIV-positive woman develops uterine cancer, for example, she is considered to have AIDS. If she is not HIV positive, she simply has uterine cancer. An HIV-positive man with tuberculosis has AIDS; if he tests negative he simply has tuberculosis. If he lives in Kenya or Colombia, where the test for HIV antibodies is too expensive, he is simply presumed to have the antibodies and therefore AIDS, and therefore he can be treated in the World Health Organization's clinic. It's the only medical help available in some places. And it's free, because the countries that support WHO are worried about AIDS.

Mullis accuses the CDC of continually adding new diseases (see ever more 'Covid symptoms') to the grand AIDS definition and of virtually doctoring the books to make it appear as if the disease continued to spread. He cites how in 1993 the CDC enormously broadened its AIDS definition and county health authorities were delighted because they received $2,500 per year from the Federal government for every reported AIDS case. Ladies and gentlemen, I have just described, via Kary Mullis, the 'Covid pandemic' of 2020 and beyond. Every element is the same and it's been pulled off in the same way by the same networks.

The 'Covid virus' exists? Okay – prove it. Er … still waiting

What Kary Mullis described with regard to 'HIV' has been repeated with 'Covid'. A claim is made that a new, or 'novel', infection has been found and the entire medical system of the world repeats that as fact exactly as they did with HIV and AIDS. No one in the mainstream asks rather relevant questions such as 'How do you know?' and 'Where is your proof?' The SARS-Cov-2 'virus' and the 'Covid-19 disease' became an overnight 'everybody-knows-that'. The origin could be debated and mulled over, but what you could not suggest was that 'SARS-Cov-2' didn't exist. That would be

ridiculous. 'Everybody knows' the 'virus' exists. Well, I didn't for one along with American proper doctors like Andrew Kaufman and Tom Cowan and long-time American proper journalist Jon Rappaport. We dared to pursue the obvious and simple question: 'Where's the evidence?' The overwhelming majority in medicine, journalism and the general public did not think to ask that. After all, *everyone knew* there was a new 'virus'. Everyone was saying so and I heard it on the BBC. Some would eventually argue that the 'deadly virus' was nothing like as deadly as claimed, but few would venture into the realms of its very existence. Had they done so they would have found that the evidence for that claim had gone AWOL as with HIV causes AIDS. In fact, not even that. For something to go AWOL it has to exist in the first place and scientific proof for a 'SARS-Cov-2' can be filed under nothing, nowhere and zilch.

Dr Andrew Kaufman is a board-certified forensic psychiatrist in New York State, a Doctor of Medicine and former Assistant Professor and Medical Director of Psychiatry at SUNY Upstate Medical University, and Medical Instructor of Hematology and Oncology at the Medical School of South Carolina. He also studied biology at the Massachusetts Institute of Technology (MIT) and trained in Psychiatry at Duke University. Kaufman is retired from allopathic medicine, but remains a consultant and educator on natural healing, I saw a video of his very early on in the 'Covid' hoax in which he questioned claims about the 'virus' in the absence of any supporting evidence and with plenty pointing the other way. I did everything I could to circulate his work which I felt was asking the pivotal questions that needed an answer. I can recommend an excellent pull-together interview he did with the website The Last Vagabond entitled *Dr Andrew Kaufman: Virus Isolation, Terrain Theory and Covid-19* and his website is andrewkaufmanmd.com. Kaufman is not only a forensic psychiatrist; he is forensic in all that he does. He always reads original scientific papers, experiments and studies instead of second-third-fourth-hand reports about the 'virus' in the media which are repeating the repeated repetition of the narrative. When he did so with the original Chinese 'virus' papers Kaufman

realised that there was no evidence of a 'SARS-Cov-2'. They had never – from the start – shown it to exist and every repeat of this claim worldwide was based on the accepted existence of proof that was nowhere to be found – see Kary Mullis and HIV. Here we go again.

Let's postulate

Kaufman discovered that the Chinese authorities immediately concluded that the cause of an illness that broke out among about 200 initial patients in Wuhan was a 'new virus' when there were no grounds to make that conclusion. The alleged 'virus' was not isolated from other genetic material in their samples and then shown through a system known as Koch's postulates to be the causative agent of the illness. The world was told that the SARS-Cov-2 'virus' caused a disease they called 'Covid-19' which had 'flu-like' symptoms and could lead to respiratory problems and pneumonia. If it wasn't so tragic it would almost be funny. *'Flu-like' symptoms'? Pneumonia? Respiratory disease?* What in *CHINA* and particularly in *Wuhan*, one of the most polluted cities in the world with a resulting epidemic of respiratory disease?? Three hundred thousand people get pneumonia in China every year and there are nearly a billion cases worldwide of 'flu-like symptoms'. These have a whole range of causes – including pollution in Wuhan – but no other possibility was credibly considered in late 2019 when the world was told there was a new and deadly 'virus'. The global prevalence of pneumonia and 'flu-like systems' gave the Cult networks unlimited potential to re-diagnose these other causes as the mythical 'Covid-19' and that is what they did from the very start. Kaufman revealed how Chinese medical and science authorities (all subordinates to the Cult-owned communist government) took genetic material from the lungs of only a few of the first patients. The material contained their own cells, bacteria, fungi and other microorganisms living in their bodies. The only way you could prove the existence of the 'virus' and its responsibility for the alleged 'Covid-19' was to isolate the virus from all the other material – a process also known as 'purification' – and

then follow the postulates sequence developed in the late 19th century by German physician and bacteriologist Robert Koch which became the 'gold standard' for connecting an alleged causation agent to a disease:

1. The microorganism (bacteria, fungus, virus, etc.) must be present in every case of the disease and all patients must have the same symptoms. It must also *not be present in healthy individuals*.

2. The microorganism must be isolated from the host with the disease. If the microorganism is a bacteria or fungus it must be grown in a pure culture. If it is a virus, it must be purified (i.e. containing no other material except the virus particles) from a clinical sample.

3. The specific disease, with all of its characteristics, must be reproduced when the infectious agent (the purified virus or a pure culture of bacteria or fungi) is inoculated into a healthy, susceptible host.

4. The microorganism must be recoverable from the experimentally infected host as in step 2.

Not one of these criteria has been met in the case of 'SARS-Cov-2' and 'Covid-19'. Not ONE. *EVER*. Robert Koch refers to bacteria and not viruses. What are called 'viral particles' are so minute (hence masks are useless by any definition) that they could only be seen after the invention of the electron microscope in the 1930s and can still only be observed through that means. American bacteriologist and virologist Thomas Milton Rivers, the so-called 'Father of Modern Virology' who was very significantly director of the Rockefeller Institute for Medical Research in the 1930s, developed a less stringent version of Koch's postulates to identify 'virus' causation known as 'Rivers criteria'. 'Covid' did not pass that process either. Some even doubt whether any 'virus' can be isolated from other particles containing genetic material in the Koch method. Freedom of Information requests in many countries asking for scientific proof that the 'Covid virus' has been purified and isolated and shown to exist have all come back with a 'we don't have that' and when this happened with a request to the UK Department of Health they added this comment:

However, outside of the scope of the [Freedom of Information Act] and on a discretionary basis, the following information has been advised to us, which may be of interest. Most infectious diseases are caused by viruses, bacteria or fungi. Some bacteria or fungi have the capacity to grow on their own in isolation, for example in colonies on a petri dish. Viruses are different in that they are what we call 'obligate pathogens' – that is, they cannot survive or reproduce without infecting a host ...

… For some diseases, it is possible to establish causation between a microorganism and a disease by isolating the pathogen from a patient, growing it in pure culture and reintroducing it to a healthy organism. These are known as 'Koch's postulates' and were developed in 1882. However, as our understanding of disease and different disease-causing agents has advanced, these are no longer the method for determining causation [Andrew Kaufman asks why in that case are there two published articles falsely claiming to satisfy Koch's postulates].

It has long been known that viral diseases cannot be identified in this way as viruses cannot be grown in 'pure culture'. When a patient is tested for a viral illness, this is normally done by looking for the presence of antigens, or viral genetic code in a host with molecular biology techniques [Kaufman asks how you could know the origin of these chemicals without having a pure culture for comparison].

For the record 'antigens' are defined so:

Invading microorganisms have antigens on their surface that the human body can recognise as being foreign – meaning not belonging to it. When the body recognises a foreign antigen, lymphocytes (white blood cells) produce antibodies, which are complementary in shape to the antigen.

Notwithstanding that this is open to question in relation to 'SARS-Cov-2' the presence of 'antibodies' can have many causes and they are found in people that are perfectly well. Kary Mullis said: 'Antibodies … had always been considered evidence of past disease, not present disease.'

'Covid' really is a *computer* 'virus'

Where the UK Department of Health statement says 'viruses' are now 'diagnosed' through a 'viral genetic code in a host with molecular biology techniques', they mean … *the PCR test* which its inventor said cannot test for infectious disease. They have no credible method of connecting a 'virus' to a disease and we will see that there is no scientific proof that any 'virus' causes any disease or there is any such thing as a 'virus' in the way that it is described. Tenacious Canadian researcher Christine Massey and her team made

some 40 Freedom of Information requests to national public health agencies in different countries asking for proof that SARS-CoV-2 has been isolated and not one of them could supply that information. Massey said of her request in Canada: 'Freedom of Information reveals Public Health Agency of Canada has no record of 'SARS-COV-2' isolation performed by anyone, anywhere, ever.' If you accept the comment from the UK Department of Health it's because they can't isolate a 'virus'. Even so many 'science' papers claimed to have isolated the 'Covid virus' until they were questioned and had to admit they hadn't. A reply from the Robert Koch Institute in Germany was typical: 'I am not aware of a paper which purified isolated SARS-CoV-2.' So what the hell was Christian Drosten and his gang using to design the 'Covid' testing protocol that has produced all the illusory Covid' cases and 'Covid' deaths when the head of the Chinese version of the CDC admitted there was a problem right from the start in that the 'virus' had never been isolated/purified? Breathe deeply: What they are calling 'Covid' is actually created by a *computer program* i.e. *they made it up* – er, that's it. They took lung fluid, with many sources of genetic material, from one single person alleged to be infected with Covid-19 by a PCR test which they *claimed*, without clear evidence, contained a 'virus'. They used several computer programs to create a model of a theoretical virus genome sequence from more than fifty-six million small sequences of RNA, each of an unknown source, assembling them like a puzzle with no known solution. The computer filled in the gaps with sequences from bits in the gene bank to make it look like a bat SARS-like coronavirus! A wave of the magic wand and poof, an in silico (computer-generated) genome, a scientific fantasy, was created. UK health researcher Dr Kevin Corbett made the same point with this analogy:

> … It's like giving you a few bones and saying that's your fish. It could be any fish. Not even a skeleton. Here's a few fragments of bones. That's your fish … It's all from gene bank and the bits of the virus sequence that weren't there they made up.
>
> They synthetically created them to fill in the blanks. That's what genetics is; it's a code. So it's ABBBCCDDD and you're missing some what you think is EEE so you put it in. It's all

synthetic. You just manufacture the bits that are missing. This is the end result of the geneticization of virology. This is basically a computer virus.

Further confirmation came in an email exchange between British citizen journalist Frances Leader and the government's Medicines & Healthcare Products Regulatory Agency (the Gates-funded MHRA) which gave emergency permission for untested 'Covid vaccines' to be used. The agency admitted that the 'vaccine' is not based on an isolated 'virus', but comes from a *computer-generated model*. Frances Leader was naturally banned from Cult-owned fascist Twitter for making this exchange public. The process of creating computer-generated alleged 'viruses' is called 'in silico' or 'in silicon' – computer chips – and the term 'in silico' is believed to originate with biological experiments using only a computer in 1989. 'Vaccines' involved with 'Covid' are also produced 'in silico' or by computer not a natural process. If the original 'virus' is nothing more than a made-up computer model how can there be 'new variants' of something that never existed in the first place? They are not new 'variants'; they are new *computer models* only minutely different to the original program and designed to further terrify the population into having the 'vaccine' and submitting to fascism. You want a 'new variant'? Click, click, enter – there you go. Tell the medical profession that you have discovered a 'South African variant', 'UK variants' or a 'Brazilian variant' and in the usual HIV-causes-AIDS manner they will unquestioningly repeat it with no evidence whatsoever to support these claims. They will go on television and warn about the dangers of 'new variants' while doing nothing more than repeating what they have been told to be true and knowing that any deviation from that would be career suicide. Big-time insiders will know it's a hoax, but much of the medical community is clueless about the way they are being played and themselves play the public without even being aware they are doing so. What an interesting 'coincidence' that AstraZeneca and Oxford University were conducting 'Covid vaccine trials' in the three countries – the UK, South Africa and Brazil – where the first three 'variants' were claimed to have 'broken out'.

Here's your 'virus' – it's a unicorn

Dr Andrew Kaufman presented a brilliant analysis describing how the 'virus' was imagined into fake existence when he dissected an article published by *Nature* and written by 19 authors detailing *alleged* 'sequencing of a complete viral genome' of the 'new SARS-CoV-2 virus'. This computer-modelled *in silico* genome was used as a template for all subsequent genome sequencing experiments that resulted in the so-called variants which he said now number more than *6,000*. The fake genome was constructed from more than 56 million individual short strands of RNA. Those little pieces were assembled into longer pieces by finding areas of overlapping sequences. The computer programs created over two million possible combinations from which the authors simply chose the longest one. They then compared this to a 'bat virus' and the computer 'alignment' rearranged the sequence and filled in the gaps! They called this computer-generated abomination the 'complete genome'. Dr Tom Cowan, a fellow medical author and collaborator with Kaufman, said such computer-generation constitutes scientific fraud and he makes this superb analogy:

> Here is an equivalency: A group of researchers claim to have found a unicorn because they found a piece of a hoof, a hair from a tail, and a snippet of a horn. They then add that information into a computer and program it to re-create the unicorn, and they then claim this computer re-creation is the real unicorn. Of course, they had never actually seen a unicorn so could not possibly have examined its genetic makeup to compare their samples with the actual unicorn's hair, hooves and horn.
>
> The researchers claim they decided which is the real genome of SARS-CoV-2 by 'consensus', sort of like a vote. Again, different computer programs will come up with different versions of the imaginary 'unicorn', so they come together as a group and decide which is the real imaginary unicorn.

This is how the 'virus' that has transformed the world was brought into fraudulent 'existence'. Extraordinary, yes, but as the Nazis said the bigger the lie the more will believe it. Cowan, however, wasn't finished and he went on to identify what he called the real blockbuster in the paper. He quotes this section from a paper written

by virologists and published by the CDC and then explains what it means:

Therefore, we examined the capacity of SARS-CoV-2 to infect and replicate in several common primate and human cell lines, including human adenocarcinoma cells (A549), human liver cells (HUH 7.0), and human embryonic kidney cells (HEK-293T). In addition to Vero E6 and Vero CCL81 cells. ... Each cell line was inoculated at high multiplicity of infection and examined 24h post-infection.

No CPE was observed in any of the cell lines except in Vero cells, which grew to greater than 10 to the 7th power at 24 h post-infection. In contrast, HUH 7.0 and 293T showed only modest viral replication, and A549 cells were incompatible with SARS CoV-2 infection.

Cowan explains that when virologists attempt to prove infection they have three possible 'hosts' or models on which they can test. The first was humans. Exposure to humans was generally not done for ethical reasons and has never been done with SARS-CoV-2 or any coronavirus. The second possible host was animals. Cowan said that forgetting for a moment that they never actually use purified virus when exposing animals they do use solutions that they *claim* contain the virus. Exposure to animals has been done with SARS-CoV-2 in an experiment involving mice and this is what they found: *None of the wild (normal) mice got sick*. In a group of genetically-modified mice, a statistically insignificant number lost weight and had slightly bristled fur, but they experienced nothing like the illness called 'Covid-19'. Cowan said the third method – the one they mostly rely on – is to inoculate solutions they *say* contain the virus onto a variety of tissue cultures. This process had never been shown to kill tissue *unless* the sample material was starved of nutrients and poisoned as *part of the process*. Yes, incredibly, in tissue experiments designed to show the 'virus' is responsible for killing the tissue they starve the tissue of nutrients and add toxic drugs including antibiotics and they do not have control studies to see if it's the starvation and poisoning that is degrading the tissue rather than the 'virus' they allege to be in there somewhere. You want me to pinch you? Yep, I understand. Tom Cowan said this about the whole nonsensical farce as he explains what that quote from the CDC paper really means:

The shocking thing about the above quote is that using their own methods, the virologists found that solutions containing SARS-CoV-2 – even in high amounts – were NOT, I repeat NOT, infective to any of the three human tissue cultures they tested. In plain English, this means they proved, on their terms, that this 'new coronavirus' is not infectious to human beings. It is ONLY infective to monkey kidney cells, and only then when you add two potent drugs (gentamicin and amphotericin), known to be toxic to kidneys, to the mix.

My friends, read this again and again. These virologists, published by the CDC, performed a clear proof, on their terms, showing that the SARS-CoV-2 virus is harmless to human beings. That is the only possible conclusion, but, unfortunately, this result is not even mentioned in their conclusion. They simply say they can provide virus stocks cultured only on monkey Vero cells, thanks for coming.

Cowan concluded: 'If people really understood how this "science" was done, I would hope they would storm the gates and demand honesty, transparency and truth.' Dr Michael Yeadon, former Vice President and Chief Scientific Adviser at drug giant Pfizer has been a vocal critic of the 'Covid vaccine' and its potential for multiple harm. He said in an interview in April, 2021, that 'not one [vaccine] has the virus. He was asked why vaccines normally using a 'dead' version of a disease to activate the immune system were not used for 'Covid' and instead we had the synthetic methods of the 'mRNA Covid vaccine'. Yeadon said that to do the former 'you'd have to have some of [the virus] wouldn't you?' He added: 'No-one's got any – seriously.' Yeadon said that surely they couldn't have fooled the whole world for a year without having a virus, 'but oddly enough ask around – no one's got it'. He didn't know why with all the 'great labs' around the world that the virus had not been isolated – 'Maybe they've been too busy running bad PCR tests and vaccines that people don't need.' What is today called 'science' is not 'science' at all. Science is no longer what is, but whatever people can be manipulated to *believe* that it is. Real science has been hijacked by the Cult to dispense and produce the 'expert scientists' and contentions that suit the agenda of the Cult. How big-time this has happened with the 'Covid' hoax which is entirely based on fake science delivered by fake 'scientists' and fake 'doctors'. The human-caused climate change hoax is also entirely based on fake science delivered by fake 'scientists' and fake 'climate experts'. In both cases real

scientists, climate experts and doctors have their views suppressed and deleted by the Cult-owned science establishment, media and Silicon Valley. This is the 'science' that politicians claim to be 'following' and a common denominator of 'Covid' and climate are Cult psychopaths Bill Gates and his mate Klaus Schwab at the Gates-funded World Economic Forum. But, don't worry, it's all just a coincidence and absolutely nothing to worry about. *Zzzzzzzz*.

What is a 'virus' REALLY?

Dr Tom Cowan is one of many contesting the very existence of viruses let alone that they cause disease. This is understandable when there is no scientific evidence for a disease-causing 'virus'. German virologist Dr Stefan Lanka won a landmark case in 2017 in the German Supreme Court over his contention that there is no such thing as a measles virus. He had offered a big prize for anyone who could prove there is and Lanka won his case when someone sought to claim the money. There is currently a prize of more than 225,000 euros on offer from an Isolate Truth Fund for anyone who can prove the isolation of SARS-CoV-2 and its genetic substance. Lanka wrote in an article headed 'The Misconception Called Virus' that scientists think a 'virus' is causing tissue to become diseased and degraded when in fact it is the *processes they are using* which do that – not a 'virus'. Lanka has done an important job in making this point clear as Cowan did in his analysis of the CDC paper. Lanka says that all claims about viruses as disease-causing pathogens are wrong and based on 'easily recognisable, understandable and verifiable misinterpretations.' Scientists believed they were working with 'viruses' in their laboratories when they were really working with 'typical particles of specific dying tissues or cells …' Lanka said that the tissue decaying process claimed to be caused by a 'virus' still happens when no alleged 'virus' is involved. It's the *process* that does the damage and not a 'virus'. The genetic sample is deprived of nutrients, removed from its energy supply through removal from the body and then doused in toxic antibiotics to remove any bacteria. He confirms again that establishment scientists do not (pinch me)

conduct control experiments to see if this is the case and if they did they would see the claims that 'viruses' are doing the damage is nonsense. He adds that during the measles 'virus' court case he commissioned an independent laboratory to perform just such a control experiment and the result was that the tissues and cells died in the exact same way as with alleged 'infected' material. This is supported by a gathering number of scientists, doctors and researchers who reject what is called 'germ theory' or the belief in the body being infected by contagious sources emitted by other people. Researchers Dawn Lester and David Parker take the same stance in their highly-detailed and sourced book *What Really Makes You Ill – Why everything you thought you knew about disease is wrong* which was recommended to me by a number of medical professionals genuinely seeking the truth. Lester and Parker say there is no provable scientific evidence to show that a 'virus' can be transmitted between people or people and animals or animals and people:

> The definition also claims that viruses are the cause of many diseases, as if this has been definitively proven. But this is not the case; there is no original scientific evidence that definitively demonstrates that any virus is the cause of any disease. The burden of proof for any theory lies with those who proposed it; but none of the existing documents provides 'proof' that supports the claim that 'viruses' are pathogens.

Dr Tom Cowan employs one of his clever analogies to describe the process by which a 'virus' is named as the culprit for a disease when what is called a 'virus' is only material released by cells detoxing themselves from infiltration by chemical or radiation poisoning. The tidal wave of technologically-generated radiation in the 'smart' modern world plus all the toxic food and drink are causing this to happen more than ever. Deluded 'scientists' misread this as a gathering impact of what they wrongly label 'viruses'.

Paper can infect houses

Cowan said in an article for davidicke.com – with his tongue only mildly in his cheek – that he believed he had made a tremendous

discovery that may revolutionise science. He had discovered that small bits of paper are alive, 'well alive-ish', can 'infect' houses, and then reproduce themselves inside the house. The result was that this explosion of growth in the paper inside the house causes the house to explode, blowing it to smithereens. His evidence for this new theory is that in the past months he had carefully examined many of the houses in his neighbourhood and found almost no scraps of paper on the lawns and surrounds of the house. There was an occasional stray label, but nothing more. Then he would return to these same houses a week or so later and with a few, not all of them, particularly the old and decrepit ones, he found to his shock and surprise they were littered with stray bits of paper. He knew then that the paper had infected these houses, made copies of itself, and blew up the house. A young boy on a bicycle at one of the sites told him he had seen a demolition crew using dynamite to explode the house the previous week, but Cowan dismissed this as the idle thoughts of silly boys because 'I was on to something big'. He was on to how 'scientists' mistake genetic material in the detoxifying process for something they call a 'virus'. Cowan said of his house and paper story:

> If this sounds crazy to you, it's because it should. This scenario is obviously nuts. But consider this admittedly embellished, for effect, current viral theory that all scientists, medical doctors and virologists currently believe.

He takes the example of the 'novel SARS-Cov2' virus to prove the point. First they take someone with an undefined illness called 'Covid-19' and don't even attempt to find any virus in their sputum. Never mind the scientists still describe how this 'virus', which they have not located attaches to a cell receptor, injects its genetic material, in 'Covid's' case, RNA, into the cell. The RNA once inserted exploits the cell to reproduce itself and makes 'thousands, nay millions, of copies of itself … Then it emerges victorious to claim its next victim':

If you were to look in the scientific literature for proof, actual scientific proof, that uniform SARS-CoV2 viruses have been properly isolated from the sputum of a sick person, that actual spike proteins could be seen protruding from the virus (which has not been found), you would find that such evidence doesn't exist.

If you go looking in the published scientific literature for actual pictures, proof, that these spike proteins or any viral proteins are ever attached to any receptor embedded in any cell membrane, you would also find that no such evidence exists. If you were to look for a video or documented evidence of the intact virus injecting its genetic material into the body of the cell, reproducing itself and then emerging victorious by budding off the cell membrane, you would find that no such evidence exists.

The closest thing you would find is electron micrograph pictures of cellular particles, possibly attached to cell debris, both of which to be seen were stained by heavy metals, a process that completely distorts their architecture within the living organism. This is like finding bits of paper stuck to the blown-up bricks, thereby proving the paper emerged by taking pieces of the bricks on its way out.

The Enders baloney

Cowan describes the 'Covid' story as being just as make-believe as his paper story and he charts back this fantasy to a Nobel Prize winner called John Enders (1897-1985), an American biomedical scientist who has been dubbed 'The Father of Modern Vaccines'. Enders is claimed to have 'discovered' the process of the viral culture which 'proved' that a 'virus' caused measles. Cowan explains how Enders did this 'by using the EXACT same procedure that has been followed by every virologist to find and characterize every new virus since 1954'. Enders took throat swabs from children with measles and immersed them in 2ml of milk. Penicillin (100u/ml) and the antibiotic streptomycin (50,g/ml) were added and the whole mix was centrifuged – rotated at high speed to separate large cellular debris from small particles and molecules as with milk and cream, for example. Cowan says that if the aim is to find little particles of genetic material ('viruses') in the snot from children with measles it would seem that the last thing you would do is mix the snot with other material – milk –that also has genetic material. 'How are you ever going to know whether whatever you found came from the snot or the milk?' He points out that streptomycin is a 'nephrotoxic' or poisonous-to-the-kidney drug. You will see the relevance of that

shortly. Cowan says that it gets worse, much worse, when Enders describes the culture medium upon which the virus 'grows': 'The culture medium consisted of bovine amniotic fluid (90%), beef embryo extract (5%), horse serum (5%), antibiotics and phenol red as an indicator of cell metabolism.' Cowan asks incredulously: 'Did he just say that the culture medium also contained fluids and tissues that are themselves rich sources of genetic material?' The genetic cocktail, or 'medium', is inoculated onto tissue and cells from rhesus monkey *kidney* tissue. This is where the importance of streptomycin comes in and currently-used antimicrobials and other drugs that are *poisonous to kidneys* and used in ALL modern viral cultures (e.g. gentamicin, streptomycin, and amphotericin). Cowan asks: 'How are you ever going to know from this witch's brew where any genetic material comes from as we now have five different sources of rich genetic material in our mix?' Remember, he says, that all genetic material, whether from monkey kidney tissues, bovine serum, milk, etc., is made from the exact same components. The same central question returns: 'How are you possibly going to know that it was the virus that killed the kidney tissue and not the toxic antibiotic and starvation rations on which you are growing the tissue?' John Enders answered the question himself – *you can't*:

A second agent was obtained from an uninoculated culture of monkey kidney cells. The cytopathic changes [death of the cells] it induced in the unstained preparations could not be distinguished with confidence from the viruses isolated from measles.

The death of the cells ('cytopathic changes') happened in exactly the same manner, whether they inoculated the kidney tissue with the measles snot or not, Cowan says. 'This is evidence that the destruction of the tissue, the very proof of viral causation of illness, was not caused by anything in the snot because they saw the same destructive effect when the snot was not even used … the cytopathic, i.e., cell-killing, changes come from the process of the culture itself, not from any virus in any snot, period.' Enders quotes in his 1957 paper a virologist called Ruckle as reporting similar findings 'and in addition has isolated an agent from monkey kidney tissue that is so

far indistinguishable from human measles virus'. In other words, Cowan says, these particles called 'measles viruses' are simply and clearly breakdown products of the starved and poisoned tissue. For measles 'virus' see all 'viruses' including the so-called 'Covid virus'. Enders, the 'Father of Modern Vaccines', also said:

There is a potential risk in employing cultures of primate cells for the production of vaccines composed of attenuated virus, since the presence of other agents possibly latent in primate tissues cannot be definitely excluded by any known method.

Cowan further quotes from a paper published in the journal *Viruses* in May, 2020, while the 'Covid pandemic' was well underway in the media if not in reality. 'EVs' here refers to particles of genetic debris from our own tissues, such as exosomes of which more in a moment: 'The remarkable resemblance between EVs and viruses has caused quite a few problems in the studies focused on the analysis of EVs released during viral infections.' Later the paper adds that to date a reliable method that can actually guarantee a complete separation (of EVs from viruses) DOES NOT EXIST. This was published at a time when a fairy tale 'virus' was claimed in total certainty to be causing a fairy tale 'viral disease' called 'Covid-19' – a fairy tale that was already well on the way to transforming human society in the image that the Cult has worked to achieve for so long. Cowan concludes his article:

To summarize, there is no scientific evidence that pathogenic viruses exist. What we think of as 'viruses' are simply the normal breakdown products of dead and dying tissues and cells. When we are well, we make fewer of these particles; when we are starved, poisoned, suffocated by wearing masks, or afraid, we make more.

There is no engineered virus circulating and making people sick. People in laboratories all over the world are making genetically modified products to make people sick. These are called vaccines. There is no virome, no 'ecosystem' of viruses, viruses are not 8%, 50% or 100 % of our genetic material. These are all simply erroneous ideas based on the misconception called a virus.

What is 'Covid'? Load of bollocks

The background described here by Cowan and Lanka was emphasised in the first video presentation that I saw by Dr Andrew Kaufman when he asked whether the 'Covid virus' was in truth a natural defence mechanism of the body called 'exosomes'. These are released by cells when in states of toxicity – see the same themes returning over and over. They are released ever more profusely as chemical and radiation toxicity increases and think of the potential effect therefore of 5G alone as its destructive frequencies infest the human energetic information field with a gathering pace (5G went online in Wuhan in 2019 as the 'virus' emerged). I'll have more about this later. Exosomes transmit a warning to the rest of the body that 'Houston, we have a problem'. Kaufman presented images of exosomes and compared them with 'Covid' under an electron microscope and the similarity was remarkable. They both attach to the same cell receptors (*claimed* in the case of 'Covid'), contain the same genetic material in the form of RNA or ribonucleic acid, and both are found in 'viral cell cultures' with damaged or dying cells. James Hildreth MD, President and Chief Executive Officer of the Meharry Medical College at Johns Hopkins, said: 'The virus is fully an exosome in every sense of the word.' Kaufman's conclusion was that there is no 'virus': 'This entire pandemic is a completely manufactured crisis … there is no evidence of anyone dying from [this] illness.' Dr Tom Cowan and Sally Fallon Morell, authors of *The Contagion Myth*, published a statement with Dr Kaufman in February, 2021, explaining why the 'virus' does not exist and you can read it that in full in the Appendix.

'Virus' theory can be traced to the 'cell theory' in 1858 of German physician Rudolf Virchow (1821-1920) who contended that disease originates from a single cell infiltrated by a 'virus'. Dr Stefan Lanka said that findings and insights with respect to the structure, function and central importance of tissues in the creation of life, which were already known in 1858, comprehensively refute the cell theory. Virchow ignored them. We have seen the part later played by John Enders in the 1950s and Lanka notes that infection theories were only established as a global dogma through the policies and

eugenics of the Third Reich in Nazi Germany (creation of the same Sabbatian cult behind the 'Covid' hoax). Lanka said: 'Before 1933, scientists dared to contradict this theory; after 1933, these critical scientists were silenced'. Dr Tom Cowan's view is that ill-heath is caused by too much of something, too little of something, or toxification from chemicals and radiation – not contagion. We must also highlight as a major source of the 'virus' theology a man still called the 'Father of Modern Virology' – Thomas Milton Rivers (1888-1962). There is no way given the Cult's long game policy that it was a coincidence for the 'Father of Modern Virology' to be director of the Rockefeller Institute for Medical Research from 1937 to 1956 when he is credited with making the Rockefeller Institute a leader in 'viral research'. Cult Rockefellers were the force behind the creation of Big Pharma 'medicine', established the World Health Organisation in 1948, and have long and close associations with the Gates family that now runs the WHO during the pandemic hoax through mega-rich Cult gofer and psychopath Bill Gates.

Only a Renegade Mind can see through all this bullshit by asking the questions that need to be answered, not taking 'no' or prevarication for an answer, and certainly not hiding from the truth in fear of speaking it. Renegade Minds have always changed the world for the better and they will change this one no matter how bleak it may currently appear to be.

CHAPTER SIX

Sequence of deceit

If you tell the truth, you don't have to remember anything
Mark Twain

Against the background that I have laid out this far the sequence that took us from an invented 'virus' in Cult-owned China in late 2019 to the fascist transformation of human society can be seen and understood in a whole new context.

We were told that a deadly disease had broken out in Wuhan and the world media began its campaign (coordinated by behavioural psychologists as we shall see) to terrify the population into unquestioning compliance. We were shown images of Chinese people collapsing in the street which never happened in the West with what was supposed to be the same condition. In the earliest days when alleged cases and deaths were few the fear register was hysterical in many areas of the media and this would expand into the common media narrative across the world. The real story was rather different, but we were never told that. The Chinese government, one of the Cult's biggest centres of global operation, said they had discovered a new illness with flu-like and pneumonia-type symptoms in a city with such toxic air that it is overwhelmed with flu-like symptoms, pneumonia and respiratory disease. Chinese scientists said it was a new – 'novel' – coronavirus which they called Sars-Cov-2 and that it caused a disease they labelled 'Covid-19'. There was no evidence for this and the 'virus' has never to this day been isolated, purified and its genetic code established from that. It

was from the beginning a computer-generated fiction. Stories of Chinese whistleblowers saying the number of deaths was being supressed or that the 'new disease' was related to the Wuhan bio-lab misdirected mainstream and alternative media into cul-de-sacs to obscure the real truth – there was no 'virus'.

Chinese scientists took genetic material from the lung fluid of just a few people and said they had found a 'new' disease when this material had a wide range of content. There was no evidence for a 'virus' for the very reasons explained in the last two chapters. The 'virus' has never been shown to (a) exist and (b) cause any disease. People were diagnosed on symptoms that are so widespread in Wuhan and polluted China and with a PCR test that can't detect infectious disease. On this farce the whole global scam was sold to the rest of the world which would also diagnose respiratory disease as 'Covid-19' from symptoms alone or with a PCR test not testing for a 'virus'. Flu miraculously disappeared *worldwide* in 2020 and into 2021 as it was redesignated 'Covid-19'. It was really the same old flu with its 'flu-like' symptoms attributed to 'flu-like' 'Covid-19'. At the same time with very few exceptions the Chinese response of draconian lockdown and fascism was the chosen weapon to respond across the West as recommended by the Cult-owned Tedros at the Cult-owned World Health Organization run by the Cult-owned Gates. All was going according to plan. Chinese scientists – everything in China is controlled by the Cult-owned government – compared their contaminated RNA lung-fluid material with other RNA sequences and said it appeared to be just under 80 percent identical to the SARS-CoV-1 'virus' claimed to be the cause of the SARS (severe acute respiratory syndrome) 'outbreak' in 2003. They decreed that because of this the 'new virus' had to be related and they called it SARS-CoV-2. There are some serious problems with this assumption and *assumption* was all it was. Most 'factual' science turns out to be assumptions repeated into everyone-knows-that. A match of under 80-percent is meaningless. Dr Kaufman makes the point that there's a *96 percent* genetic correlation between humans and chimpanzees, but 'no one would say our genetic material is part

of the chimpanzee family'. Yet the Chinese authorities were claiming that a much lower percentage, less than 80 percent, proved the existence of a new 'coronavirus'. For goodness sake human DNA is 60 percent similar to a *banana*.

You are feeling sleepy

The entire 'Covid' hoax is a global Psyop, a psychological operation to program the human mind into believing and fearing a complete fantasy. A crucial aspect of this was what *appeared* to happen in Italy. It was all very well streaming out daily images of an alleged catastrophe in Wuhan, but to the Western mind it was still on the other side of the world in a very different culture and setting. A reaction of 'this could happen to me and my family' was still nothing like as intense enough for the mind-doctors. The Cult needed a Western example to push people over that edge and it chose Italy, one of its major global locations going back to the Roman Empire. An Italian 'Covid' crisis was manufactured in a particular area called Lombardy which just happens to be notorious for its toxic air and therefore respiratory disease. Wuhan, China, déjà vu. An hysterical media told horror stories of Italians dying from 'Covid' in their droves and how Lombardy hospitals were being overrun by a tidal wave of desperately ill people needing treatment after being struck down by the 'deadly virus'. Here was the psychological turning point the Cult had planned. Wow, if this is happening in Italy, the Western mind concluded, this indeed could happen to me and my family. Another point is that Italian authorities responded by following the Chinese blueprint so vehemently recommended by the Cult-owned World Health Organization. They imposed fascistic lockdowns on the whole country viciously policed with the help of surveillance drones sweeping through the streets seeking out anyone who escaped from mass house arrest. Livelihoods were destroyed and psychology unravelled in the way we have witnessed since in all lockdown countries. Crucial to the plan was that Italy responded in this way to set the precedent of suspending freedom and imposing fascism in a 'Western liberal democracy'. I emphasised in an

animated video explanation on davidicke.com posted in the summer of 2020 how important it was to the Cult to expand the Chinese lockdown model across the West. Without this, and the bare-faced lie that non-symptomatic people could still transmit a 'disease' they didn't have, there was no way locking down the whole population, sick and not sick, could be pulled off. At just the right time and with no evidence Cult operatives and gofers claimed that people without symptoms could pass on the 'disease'. In the name of protecting the 'vulnerable' like elderly people, who lockdowns would kill by the tens of thousands, we had for the first time healthy people told to isolate as well as the sick. The great majority of people who tested positive had no symptoms because there was nothing wrong with them. It was just a trick made possible by a test not testing for the 'virus'.

Months after my animated video the Gates-funded Professor Neil Ferguson at the Gates-funded Imperial College confirmed that I was right. He didn't say it in those terms, naturally, but he did say it. Ferguson will enter the story shortly for his outrageously crazy 'computer models' that led to Britain, the United States and many other countries following the Chinese and now Italian methods of response. Put another way, following the Cult script. Ferguson said that SAGE, the UK government's scientific advisory group which has controlled 'Covid' policy from the start, wanted to follow the Chinese lockdown model (while they all continued to work and be paid), but they wondered if they could possibly, in Ferguson's words, 'get away with it in Europe'. 'Get away with it'? Who the hell do these moronic, arrogant people think they are? This appalling man Ferguson said that once Italy went into national lockdown they realised they, too, could mimic China:

> It's a communist one-party state, we said. We couldn't get away with it in Europe, we thought … and then Italy did it. And we realised we could. Behind this garbage from Ferguson is a simple fact: Doing the same as China in every country was the plan from the start and Ferguson's 'models' would play a central role in achieving that. It's just a coincidence, of course, and absolutely nothing to worry your little head about.

Oops, sorry, our mistake

Once the Italian segment of the Psyop had done the job it was designed to do a very different story emerged. Italian authorities revealed that 99 percent of those who had 'died from Covid-19' in Italy had one, two, three, or more 'co-morbidities' or illnesses and health problems that could have ended their life. The US Centers for Disease Control and Prevention (CDC) published a figure of 94 percent for Americans dying of 'Covid' while having other serious medical conditions – on average two to three (some five or six) other potential causes of death. In terms of death from an unproven 'virus' I say it is 100 percent. The other one percent in Italy and six percent in the US would presumably have died from 'Covid's' flu-like symptoms with a range of other possible causes in conjunction with a test not testing for the 'virus'. Fox News reported that even more startling figures had emerged in one US county in which 410 of 422 deaths attributed to 'Covid-19' had other potentially deadly health conditions. The Italian National Health Institute said later that the average age of people dying with a 'Covid-19' diagnosis in Italy was about 81. Ninety percent were over 70 with ten percent over 90. In terms of other reasons to die some 80 percent had two or more chronic diseases with half having three or more including cardiovascular problems, diabetes, respiratory problems and cancer. Why is the phantom 'Covid-19' said to kill overwhelmingly old people and hardly affect the young? Old people continually die of many causes and especially respiratory disease which you can re-diagnose 'Covid-19' while young people die in tiny numbers by comparison and rarely of respiratory disease. Old people 'die of Covid' because they die of other things that can be redesignated 'Covid' and it really is that simple.

Flu has flown

The blueprint was in place. Get your illusory 'cases' from a test not testing for the 'virus' and redesignate other causes of death as 'Covid-19'. You have an instant 'pandemic' from something that is nothing more than a computer-generated fiction. With near-on a

billion people having 'flu-like' symptoms every year the potential was limitless and we can see why flu quickly and apparently miraculously disappeared *worldwide* by being diagnosed 'Covid-19'. The painfully bloody obvious was explained away by the childlike media in headlines like this in the UK '*Independent*': 'Not a single case of flu detected by Public Health England this year as Covid restrictions suppress virus'. I kid you not. The masking, social distancing and house arrest that did not make the 'Covid virus' disappear somehow did so with the 'flu virus'. Even worse the article, by a bloke called Samuel Lovett, suggested that maybe the masking, sanitising and other 'Covid' measures should continue to keep the flu away. With a ridiculousness that disturbs your breathing (it's 'Covid-19') the said Lovett wrote: 'With widespread social distancing and mask-wearing measures in place throughout the UK, the usual routes of transmission for influenza have been blocked.' He had absolutely no evidence to support that statement, but look at the consequences of him acknowledging the obvious. With flu not disappearing at all and only being relabelled 'Covid-19' he would have to contemplate that 'Covid' was a hoax on a scale that is hard to imagine. You need guts and commitment to truth to even go there and that's clearly something Samuel Lovett does not have in abundance. He would never have got it through the editors anyway.

Tens of thousands die in the United States alone every winter from flu including many with pneumonia complications. CDC figures record *45 million* Americans diagnosed with flu in 2017-2018 of which 61,000 died and some reports claim 80,000. Where was the same hysteria then that we have seen with 'Covid-19'? Some 250,000 Americans are admitted to hospital with pneumonia every year with about 50,000 cases proving fatal. About 65 million suffer respiratory disease every year and three million deaths makes this the third biggest cause of death worldwide. You only have to redesignate a portion of all these people 'Covid-19' and you have an instant global pandemic or the *appearance* of one. Why would doctors do this? They are told to do this and all but a few dare not refuse those who must be obeyed. Doctors in general are not researching their own

knowledge and instead take it direct and unquestioned from the authorities that own them and their careers. The authorities say they must now diagnose these symptoms 'Covid-19' and not flu, or whatever, and they do it. Dark suits say put 'Covid-19' on death certificates no matter what the cause of death and the doctors do it. Renegade Minds don't fall for the illusion that doctors and medical staff are all highly-intelligent, highly-principled, seekers of medical truth. *Some are*, but not the majority. They are repeaters, gofers, and yes sir, no sir, purveyors of what the system demands they purvey. The 'Covid' con is not merely confined to diseases of the lungs. Instructions to doctors to put 'Covid-19' on death certificates for anyone dying of *anything* within 28 days (or much more) of a positive test not testing for the 'virus' opened the floodgates. The term dying *with* 'Covid' and not *of* 'Covid' was coined to cover the truth. Whether it was a *with* or an *of* they were all added to the death numbers attributed to the 'deadly virus' compiled by national governments and globally by the Gates-funded Johns Hopkins operation in the United States that was so involved in those 'pandemic' simulations. Fraudulent deaths were added to the ever-growing list of fraudulent 'cases' from false positives from a false test. No wonder Professor Walter Ricciardi, scientific advisor to the Italian minister of health, said after the Lombardy hysteria had done its job that 'Covid' death rates were due to Italy having the second oldest population in the world and to *how hospitals record deaths*:

The way in which we code deaths in our country is very generous in the sense that all the people who die in hospitals with the coronavirus are deemed to be dying of the coronavirus. On re-evaluation by the National Institute of Health, only 12 per cent of death certificates have shown a direct causality from coronavirus, while 88 per cent of patients who have died have at least one pre-morbidity – many had two or three.

This is extraordinary enough when you consider the propaganda campaign to use Italy to terrify the world, but how can they even say twelve percent were genuine when the 'virus' has not been shown to exist, its 'code' is a computer program, and diagnosis comes from a test not testing for it? As in China, and soon the world, 'Covid-19' in

Italy was a redesignation of diagnosis. Lies and corruption were to become the real 'pandemic' fuelled by a pathetically-compliant medical system taking its orders from the tiny few at the top of their national hierarchy who answered to the World Health Organization which answers to Gates and the Cult. Doctors were told – ordered – to diagnose a particular set of symptoms 'Covid-19' and put that on the death certificate for any cause of death if the patient had tested positive with a test not testing for the virus or had 'Covid' symptoms like the flu. The United States even introduced big financial incentives to manipulate the figures with hospitals receiving £4,600 from the Medicare system for diagnosing someone with regular pneumonia, $13,000 if they made the diagnosis from the same symptoms 'Covid-19' pneumonia, and $39, 000 if they put a 'Covid' diagnosed patient on a ventilator that would almost certainly kill them. A few – painfully and pathetically few – medical whistleblowers revealed (before Cult-owned YouTube deleted their videos) that they had been instructed to 'let the patient crash' and put them straight on a ventilator instead of going through a series of far less intrusive and dangerous methods as they would have done before the pandemic hoax began and the financial incentives kicked in. We are talking cold-blooded murder given that ventilators are so damaging to respiratory systems they are usually the last step before heaven awaits. Renegade Minds never fall for the belief that people in white coats are all angels of mercy and cannot be full-on psychopaths. I have explained in detail in *The Answer* how what I am describing here played out across the world coordinated by the World Health Organization through the medical hierarchies in almost every country.

Medical scientist calls it

Information about the non-existence of the 'virus' began to emerge for me in late March, 2020, and mushroomed after that. I was sent an email by Sir Julian Rose, a writer, researcher, and organic farming promotor, from a medical scientist friend of his in the United States. Even at that early stage in March the scientist was able to explain

how the 'Covid' hoax was being manipulated. He said there were no reliable tests for a specific 'Covid-19 virus' and nor were there any reliable agencies or media outlets for reporting numbers of actual 'Covid-19' cases. We have seen in the long period since then that he was absolutely right. 'Every action and reaction to Covid-19 is based on totally flawed data and we simply cannot make accurate assessments,' he said. Most people diagnosed with 'Covid-19' were showing nothing more than cold and flu-like symptoms 'because most coronavirus strains *are* nothing more than cold/flu-like symptoms'. We had farcical situations like an 84-year-old German man testing positive for 'Covid-19' and his nursing home ordered to quarantine only for him to be found to have a common cold. The scientist described back then why PCR tests and what he called the 'Mickey Mouse test kits' were useless for what they were claimed to be identifying. 'The idea these kits can isolate a specific virus like Covid-19 is nonsense,' he said. Significantly, he pointed out that 'if you want to create a totally false panic about a totally false pandemic – pick a coronavirus'. This is exactly what the Cult-owned Gates, World Economic Forum and Johns Hopkins University did with their Event 201 'simulation' followed by their real-life simulation called the 'pandemic'. The scientist said that all you had to do was select the sickest of people with respiratory-type diseases in a single location – 'say Wuhan' – and administer PCR tests to them. You can then claim that anyone showing 'viral sequences' similar to a coronavirus 'which will inevitably be quite a few' is suffering from a 'new' disease:

> Since you already selected the sickest flu cases a fairly high proportion of your sample will go on to die. You can then say this 'new' virus has a CFR [case fatality rate] higher than the flu and use this to infuse more concern and do more tests which will of course produce more 'cases', which expands the testing, which produces yet more 'cases' and so on and so on. Before long you have your 'pandemic', and all you have done is use a simple test kit trick to convert the worst flu and pneumonia cases into something new that doesn't ACTUALLY EXIST [my emphasis].

He said that you then 'just run the same scam in other countries' and make sure to keep the fear message running high 'so that people

will feel panicky and less able to think critically'. The only problem to overcome was the fact *there is no* actual new deadly pathogen and only regular sick people. This meant that deaths from the 'new deadly pathogen' were going to be way too low for a real new deadly virus pandemic, but he said this could be overcome in the following ways – all of which would go on to happen:

1. You can claim this is just the beginning and more deaths are imminent [you underpin this with fantasy 'computer projections']. Use this as an excuse to quarantine everyone and then claim the quarantine prevented the expected millions of dead.

2. You can [say that people] 'minimizing' the dangers are irresponsible and bully them into not talking about numbers.

3. You can talk crap about made up numbers hoping to blind people with pseudoscience.

4. You can start testing well people (who, of course, will also likely have shreds of coronavirus [RNA] in them) and thus inflate your 'case figures' with 'asymptomatic carriers' (you will of course have to spin that to sound deadly even though any virologist knows the more symptom-less cases you have the less deadly is your pathogen).

The scientist said that if you take these simple steps 'you can have your own entirely manufactured pandemic up and running in weeks'. His analysis made so early in the hoax was brilliantly prophetic of what would actually unfold. Pulling all the information together in these recent chapters we have this is simple 1, 2, 3, of how you can delude virtually the entire human population into believing in a 'virus' that doesn't exist:

- A 'Covid case' is someone who tests positive with a test not testing for the 'virus'.

- A 'Covid death' is someone who dies of *any cause* within 28 days (or much longer) of testing positive with a test not testing for the 'virus.

- Asymptomatic means there is nothing wrong with you, but they claim you can pass on what you don't have to justify locking

down (quarantining) healthy people in totality.

The foundations of the hoax are that simple. A study involving ten million people in Wuhan, published in November, 2020, demolished the whole lie about those without symptoms passing on the 'virus'. They found '300 asymptomatic cases' and traced their contacts to find that not one of them was detected with the 'virus'. 'Asymptomatic' patients and their contacts were isolated for no less than two weeks and nothing changed. I know it's all crap, but if you are going to claim that those without symptoms can transmit 'the virus' then you must produce evidence for that and they never have. Even World Health Organization official Dr Maria Van Kerkhove, head of the emerging diseases and zoonosis unit, said as early as June, 2020, that she doubted the validity of asymptomatic transmission. She said that 'from the data we have, it still seems to be rare that an asymptomatic person actually transmits onward to a secondary individual' and by 'rare' she meant that she couldn't cite any case of asymptomatic transmission.

The Ferguson factor

The problem for the Cult as it headed into March, 2020, when the script had lockdown due to start, was that despite all the manipulation of the case and death figures they still did not have enough people alleged to have died from 'Covid' to justify mass house arrest. This was overcome in the way the scientist described: 'You can claim this is just the beginning and more deaths are imminent … Use this as an excuse to quarantine everyone and then claim the quarantine prevented the expected millions of dead.' Enter one Professor Neil Ferguson, the Gates-funded 'epidemiologist' at the Gates-funded Imperial College in London. Ferguson is Britain's Christian Drosten in that he has a dire record of predicting health outcomes, but is still called upon to advise government on the next health outcome when another 'crisis' comes along. This may seem to be a strange and ridiculous thing to do. Why would you keep turning for policy guidance to people who have a history of being

monumentally wrong? Ah, but it makes sense from the Cult point of view. These 'experts' keep on producing predictions that suit the Cult agenda for societal transformation and so it was with Neil Ferguson as he revealed his horrific (and clearly insane) computer model predictions that allowed lockdowns to be imposed in Britain, the United States and many other countries. Ferguson does not have even an A-level in biology and would appear to have no formal training in computer modelling, medicine or epidemiology, according to Derek Winton, an MSc in Computational Intelligence. He wrote an article somewhat aghast at what Ferguson did which included taking no account of respiratory disease 'seasonality' which means it is far worse in the winter months. Who would have thought that respiratory disease could be worse in the winter? Well, certainly not Ferguson.

The massively China-connected Imperial College and its bizarre professor provided the excuse for the long-incubated Chinese model of human control to travel westward at lightning speed. Imperial College confirms on its website that it collaborates with the Chinese Research Institute; publishes more than 600 research papers every year with Chinese research institutions; has 225 Chinese staff; 2,600 Chinese students – the biggest international group; 7,000 former students living in China which is the largest group outside the UK; and was selected for a tour by China's President Xi Jinping during his state visit to the UK in 2015. The college takes major donations from China and describes itself as the UK's number one university collaborator with Chinese research institutions. The China communist/fascist government did not appear phased by the woeful predictions of Ferguson and Imperial when during the lockdown that Ferguson induced the college signed a five-year collaboration deal with China tech giant Huawei that will have Huawei's indoor 5G network equipment installed at the college's West London tech campus along with an 'AI cloud platform'. The deal includes Chinese sponsorship of Imperial's Venture Catalyst entrepreneurship competition. Imperial is an example of the enormous influence the Chinese government has within British and North American

universities and research centres – and further afield. Up to 200 academics from more than a dozen UK universities are being investigated on suspicion of 'unintentionally' helping the Chinese government build weapons of mass destruction by 'transferring world-leading research in advanced military technology such as aircraft, missile designs and cyberweapons'. Similar scandals have broken in the United States, but it's all a coincidence. Imperial College serves the agenda in many other ways including the promotion of every aspect of the United Nations Agenda 21/2030 (the Great Reset) and produced computer models to show that human-caused 'climate change' is happening when in the real world it isn't. Imperial College is driving the climate agenda as it drives the 'Covid' agenda (both Cult hoaxes) while Patrick Vallance, the UK government's Chief Scientific Adviser on 'Covid', was named Chief Scientific Adviser to the UN 'climate change' conference known as COP26 hosted by the government in Glasgow, Scotland. 'Covid' and 'climate' are fundamentally connected.

Professor Woeful

From Imperial's bosom came Neil Ferguson still advising government despite his previous disasters and it was announced early on that he and other key people like UK Chief Medical Adviser Chris Whitty had caught the 'virus' as the propaganda story was being sold. Somehow they managed to survive and we had Prime Minister Boris Johnson admitted to hospital with what was said to be a severe version of the 'virus' in this same period. His whole policy and demeanour changed when he returned to Downing Street. It's a small world with these government advisors – especially in their communal connections to Gates – and Ferguson had partnered with Whitty to write a paper called 'Infectious disease: Tough choices to reduce Ebola transmission' which involved another scare-story that didn't happen. Ferguson's 'models' predicted that up to150, 000 could die from 'mad cow disease', or BSE, and its version in sheep if it was transmitted to humans. BSE was not transmitted and instead triggered by an organophosphate pesticide used to treat a pest on

cows. Fewer than 200 deaths followed from the human form. Models by Ferguson and his fellow incompetents led to the unnecessary culling of millions of pigs, cattle and sheep in the foot and mouth outbreak in 2001 which destroyed the lives and livelihoods of farmers and their families who had often spent decades building their herds and flocks. Vast numbers of these animals did not have foot and mouth and had no contact with the infection. Another 'expert' behind the cull was Professor Roy Anderson, a computer modeller at Imperial College specialising in the epidemiology of *human*, not animal, disease. Anderson has served on the Bill and Melinda Gates Grand Challenges in Global Health advisory board and chairs another Gates-funded organisation. Gates is everywhere.

In a precursor to the 'Covid' script Ferguson backed closing schools 'for prolonged periods' over the swine flu 'pandemic' in 2009 and said it would affect a third of the world population if it continued to spread at the speed he claimed to be happening. His mates at Imperial College said much the same and a news report said: 'One of the authors, the epidemiologist and disease modeller Neil Ferguson, who sits on the World Health Organisation's emergency committee for the outbreak, said the virus had "full pandemic potential".' Professor Liam Donaldson, the Chris Whitty of his day as Chief Medical Officer, said the worst case could see 30 percent of the British people infected by swine flu with 65,000 dying. Ferguson and Donaldson were indeed proved correct when at the end of the year the number of deaths attributed to swine flu was 392. The term 'expert' is rather liberally applied unfortunately, not least to complete idiots. Swine flu 'projections' were great for GlaxoSmithKline (GSK) as millions rolled in for its Pandemrix influenza vaccine which led to brain damage with children most affected. The British government (taxpayers) paid out more than £60 million in compensation after GSK was given immunity from prosecution. Yet another 'Covid' déjà vu. Swine flu was supposed to have broken out in Mexico, but Dr Wolfgang Wodarg, a German doctor, former member of parliament and critic of the 'Covid' hoax, observed 'the spread of swine flu' in Mexico City at the time. He

said: 'What we experienced in Mexico City was a very mild flu which did not kill more than usual – which killed even fewer people than usual.' Hyping the fear against all the facts is not unique to 'Covid' and has happened many times before. Ferguson is reported to have over-estimated the projected death toll of bird flu (H5N1) by some three million-fold, but bird flu vaccine makers again made a killing from the scare. This is some of the background to the Neil Ferguson who produced the perfectly-timed computer models in early 2020 predicting that half a million people would die in Britain without draconian lockdown and 2.2 million in the United States. Politicians panicked, people panicked, and lockdowns of alleged short duration were instigated to 'flatten the curve' of cases gleaned from a test not testing for the 'virus'. I said at the time that the public could forget the 'short duration' bit. This was an agenda to destroy the livelihoods of the population and force them into mass control through dependency and there was going to be nothing 'short' about it. American researcher Daniel Horowitz described the consequences of the 'models' spewed out by Gates-funded Ferguson and Imperial College:

> What led our government and the governments of many other countries into panic was a single Imperial College of UK study, funded by global warming activists, that predicted 2.2 million deaths if we didn't lock down the country. In addition, the reported 8-9% death rate in Italy scared us into thinking there was some other mutation of this virus that they got, which might have come here.
>
> Together with the fact that we were finally testing and had the ability to actually report new cases, we thought we were headed for a death spiral. But again … we can't flatten a curve if we don't know when the curve started.

How about it *never* started?

Giving them what they want

An investigation by German news outlet *Welt Am Sonntag (World on Sunday)* revealed how in March, 2020, the German government gathered together 'leading scientists from several research institutes and universities' and 'together, they were to produce a [modelling]

paper that would serve as legitimization for further tough political measures'. The Cult agenda was justified by computer modelling not based on evidence or reality; it was specifically constructed to justify the Cult demand for lockdowns all over the world to destroy the independent livelihoods of the global population. All these modellers and everyone responsible for the 'Covid' hoax have a date with a trial like those in Nuremberg after World War Two when Nazis faced the consequences of their war crimes. These corrupt-beyond-belief 'modellers' wrote the paper according to government instructions and it said that that if lockdown measures were lifted then up to one million Germans would die from 'Covid-19' adding that some would die 'agonizingly at home, gasping for breath' unable to be treated by hospitals that couldn't cope. All lies. No matter – it gave the Cult all that it wanted. What did long-time government 'modeller' Neil Ferguson say? If the UK and the United States didn't lockdown half a million would die in Britain and 2.2 million Americans. Anyone see a theme here? 'Modellers' are such a crucial part of the lockdown strategy that we should look into their background and follow the money. Researcher Rosemary Frei produced an excellent article headlined 'The Modelling-paper Mafiosi'. She highlights a guy called John Edmunds, a British epidemiologist, and professor in the Faculty of Epidemiology and Population Health at the London School of Hygiene & Tropical Medicine. He studied at Imperial College. Edmunds is a member of government 'Covid' advisory bodies which have been dictating policy, the New and Emerging Respiratory Virus Threats Advisory Group (NERVTAG) and the Scientific Advisory Group for Emergencies (SAGE).

Ferguson, another member of NERVTAG and SAGE, led the way with the original 'virus' and Edmunds has followed in the 'variant' stage and especially the so-called UK or Kent variant known as the 'Variant of Concern' (VOC) B.1.1.7. He said in a co-written report for the Centre for Mathematical modelling of Infectious Diseases at the London School of Hygiene and Tropical Medicine, with input from the Centre's 'Covid-19' Working Group, that there was 'a realistic

possibility that VOC B.1.1.7 is associated with an increased risk of death compared to non-VOC viruses'. Fear, fear, fear, get the vaccine, fear, fear, fear, get the vaccine. Rosemary Frei reveals that almost all the paper's authors and members of the modelling centre's 'Covid-19' Working Group receive funding from the Bill and Melinda Gates Foundation and/or the associated Gates-funded Wellcome Trust. The paper was published by e-journal *Medr χiv* which only publishes papers not peer-reviewed and the journal was established by an organisation headed by Facebook's Mark Zuckerberg and his missus. What a small world it is. Frei discovered that Edmunds is on the Scientific Advisory Board of the Coalition for Epidemic Preparedness Innovations (CEPI) which was established by the Bill and Melinda Gates Foundation, Klaus Schwab's Davos World Economic Forum and Big Pharma giant Wellcome. CEPI was 'launched in Davos [in 2017] to develop vaccines to stop future epidemics', according to its website. 'Our mission is to accelerate the development of vaccines against emerging infectious diseases and enable equitable access to these vaccines for people during outbreaks.' What kind people they are. Rosemary Frei reveals that Public Health England (PHE) director Susan Hopkins is an author of her organisation's non-peer-reviewed reports on 'new variants'. Hopkins is a professor of infectious diseases at London's Imperial College which is gifted tens of millions of dollars a year by the Bill and Melinda Gates Foundation. Gates-funded modelling disaster Neil Ferguson also co-authors Public Health England reports and he spoke in December, 2020, about the potential danger of the B.1.1.7. 'UK variant' promoted by Gates-funded modeller John Edmunds. When I come to the 'Covid vaccines' the 'new variants' will be shown for what they are – bollocks.

Connections, connections

All these people and modellers are lockdown-obsessed or, put another way, they demand what the Cult demands. Edmunds said in January, 2021, that to ease lockdowns too soon would be a disaster and they had to 'vaccinate much, much, much more widely than the

elderly'. Rosemary Frei highlights that Edmunds is married to Jeanne Pimenta who is described in a LinkedIn profile as director of epidemiology at GlaxoSmithKline (GSK) and she held shares in the company. Patrick Vallance, co-chair of SAGE and the government's Chief Scientific Adviser, is a former executive of GSK and has a deferred bonus of shares in the company worth £600,000. GSK has serious business connections with Bill Gates and is collaborating with mRNA-'vaccine' company CureVac to make 'vaccines' for the new variants that Edmunds is talking about. GSK is planning a 'Covid vaccine' with drug giant Sanofi. Puppet Prime Minister Boris Johnson announced in the spring of 2021 that up to 60 million vaccine doses were to be made at the GSK facility at Barnard Castle in the English North East. Barnard Castle, with a population of just 6,000, was famously visited in breach of lockdown rules in April, 2020, by Johnson aide Dominic Cummings who said that he drove there 'to test his eyesight' before driving back to London. Cummings would be better advised to test his integrity – not that it would take long. The GSK facility had nothing to do with his visit then although I'm sure Patrick Vallance would have been happy to arrange an introduction and some tea and biscuits. Ruthless psychopath Gates has made yet another fortune from vaccines in collaboration with Big Pharma companies and gushes at the phenomenal profits to be made from vaccines – more than a 20-to-1 return as he told one interviewer. Gates also tweeted in December, 2019, with the foreknowledge of what was coming: 'What's next for our foundation? I'm particularly excited about what the next year could mean for one of the best buys in global health: vaccines.'

Modeller John Edmunds is a big promotor of vaccines as all these people appear to be. He's the dean of the London School of Hygiene & Tropical Medicine's Faculty of Epidemiology and Population Health which is primarily funded by the Bill and Melinda Gates Foundation and the Gates-established and funded GAVI vaccine alliance which is the Gates vehicle to vaccinate the world. The organisation Doctors Without Borders has described GAVI as being 'aimed more at supporting drug-industry desires to promote new

products than at finding the most efficient and sustainable means for fighting the diseases of poverty'. But then that's why the psychopath Gates created it. John Edmunds said in a video that the London School of Hygiene & Tropical Medicine is involved in every aspect of vaccine development including large-scale clinical trials. He contends that mathematical modelling can show that vaccines protect individuals and society. That's on the basis of shit in and shit out, I take it. Edmunds serves on the UK Vaccine Network as does Ferguson and the government's foremost 'Covid' adviser, the grim-faced, dark-eyed Chris Whitty. The Vaccine Network says it works 'to support the government to identify and shortlist targeted investment opportunities for the most promising vaccines and vaccine technologies that will help combat infectious diseases with epidemic potential, and to address structural issues related to the UK's broader vaccine infrastructure'. Ferguson is acting Director of the Imperial College Vaccine Impact Modelling Consortium which has funding from the Bill and Melina Gates Foundation and the Gates-created GAVI 'vaccine alliance'. Anyone wonder why these characters see vaccines as the answer to every problem? Ferguson is wildly enthusiastic in his support for GAVI's campaign to vaccine children en masse in poor countries. You would expect someone like Gates who has constantly talked about the need to reduce the population to want to fund vaccines to keep more people alive. I'm sure that's why he does it. The John Edmunds London School of Hygiene & Tropical Medicine (LSHTM) has a Vaccines Manufacturing Innovation Centre which develops, tests and commercialises vaccines. Rosemary Frei writes:

> The vaccines centre also performs affiliated activities like combating 'vaccine hesitancy'. The latter includes the Vaccine Confidence Project. The project's stated purpose is, among other things, 'to provide analysis and guidance for early response and engagement with the public to ensure sustained confidence in vaccines and immunisation'. The Vaccine Confidence Project's director is LSHTM professor Heidi Larson. For more than a decade she's been researching how to combat vaccine hesitancy.

How the bloody hell can blokes like John Edmunds and Neil Ferguson with those connections and financial ties model 'virus' case

and death projections for the government and especially in a way that gives their paymasters like Gates exactly what they want? It's insane, but this is what you find throughout the world.

'Covid' is not dangerous, oops, wait, yes it is

Only days before Ferguson's nightmare scenario made Jackboot Johnson take Britain into a China-style lockdown to save us from a deadly 'virus' the UK government website gov.uk was reporting something very different to Ferguson on a page of official government guidance for 'high consequence infectious diseases (HCID)'. It said this about 'Covid-19':

As of 19 March 2020, COVID-19 *is no longer considered to be a high consequence infectious diseases (HCID) in the UK* [my emphasis]. The 4 nations public health HCID group made an interim recommendation in January 2020 to classify COVID-19 as an HCID. This was based on consideration of the UK HCID criteria about the virus and the disease with information available during the early stages of the outbreak.

Now that more is known about COVID-19, the public health bodies in the UK have reviewed the most up to date information about COVID-19 against the UK HCID criteria. They have determined that several features have now changed; in particular, more information is available about mortality rates (low overall), and there is now greater clinical awareness and a specific and sensitive laboratory test, the availability of which continues to increase. The Advisory Committee on Dangerous Pathogens (ACDP) is also of the opinion that COVID-19 should no longer be classified as an HCID.

Soon after the government had been exposed for downgrading the risk they upgraded it again and everyone was back to singing from the same Cult hymn book. Ferguson and his fellow Gates clones indicated that lockdowns and restrictions would have to continue until a Gates-funded vaccine was developed. Gates said the same because Ferguson and his like were repeating the Gates script which is the Cult script. 'Flatten the curve' became an ongoing nightmare of continuing lockdowns with periods in between of severe restrictions in pursuit of destroying independent incomes and had nothing to do with protecting health about which the Cult gives not a shit. Why wouldn't Ferguson be pushing a vaccine 'solution' when he's owned by vaccine-obsessive Gates who makes a fortune from them and

when Ferguson heads the Vaccine Impact Modelling Consortium at Imperial College funded by the Gates Foundation and GAVI, the 'vaccine alliance', created by Gates as his personal vaccine promotion operation? To compound the human catastrophe that Ferguson's 'models' did so much to create he was later exposed for breaking his own lockdown rules by having sexual liaisons with his married girlfriend Antonia Staats at his home while she was living at another location with her husband and children. Staats was a 'climate' activist and senior campaigner at the Soros-funded Avaaz which I wouldn't trust to tell me that grass is green. Ferguson had to resign as a government advisor over this hypocrisy in May, 2020, but after a period of quiet he was back being quoted by the ridiculous media on the need for more lockdowns and a vaccine rollout. Other government-advising 'scientists' from Imperial College' held the fort in his absence and said lockdown could be indefinite until a vaccine was found. The Cult script was being sung by the payrolled choir. I said there was no intention of going back to 'normal' when the 'vaccine' came because the 'vaccine' is part of a very different agenda that I will discuss in Human 2.0. Why would the Cult want to let the world go back to normal when destroying that normal forever was the whole point of what was happening? House arrest, closing businesses and schools through lockdown, (un)social distancing and masks all followed the Ferguson fantasy models. Again as I predicted (these people are so predictable) when the 'vaccine' arrived we were told that house arrest, lockdown, (un)social distancing and masks would still have to continue. I will deal with the masks in the next chapter because they are of fundamental importance.

Where's the 'pandemic'?

Any mildly in-depth assessment of the figures revealed what was really going on. Cult-funded and controlled organisations still have genuine people working within them such is the number involved. So it is with Genevieve Briand, assistant program director of the Applied Economics master's degree program at Johns Hopkins

University. She analysed the impact that 'Covid-19' had on deaths from *all* causes in the United States using official data from the CDC for the period from early February to early September, 2020. She found that allegedly 'Covid' *related*-deaths exceeded those from heart disease which she found strange with heart disease always the biggest cause of fatalities. Her research became even more significant when she noted the sudden decline in 2020 of *all* non-'Covid' deaths: 'This trend is completely contrary to the pattern observed in all previous years … the total decrease in deaths by other causes almost exactly equals the increase in deaths by Covid-19.' This was such a game, set and match in terms of what was happening that Johns Hopkins University deleted the article on the grounds that it 'was being used to support false and dangerous inaccuracies about the impact of the pandemic'. No – because it exposed the scam from official CDC figures and this was confirmed when those figures were published in January, 2021. Here we can see the effect of people dying from heart attacks, cancer, road accidents and gunshot wounds – *anything* – having 'Covid-19' on the death certificate along with those diagnosed from 'symptoms' who had even not tested positive with a test not testing for the 'virus'. I am not kidding with the gunshot wounds, by the way. Brenda Bock, coroner in Grand County, Colorado, revealed that two gunshot victims tested positive for the 'virus' within the previous 30 days and were therefore classified as 'Covid deaths'. Bock said: 'These two people had tested positive for Covid, but that's not what killed them. A gunshot wound is what killed them.' She said she had not even finished her investigation when the state listed the gunshot victims as deaths due to the 'virus'. The death and case figures for 'Covid-19' are an absolute joke and yet they are repeated like parrots by the media, politicians and alleged medical 'experts'. The official Cult narrative is the only show in town.

Genevieve Briand found that deaths from all causes were not exceptional in 2020 compared with previous years and a Spanish magazine published figures that said the same about Spain which was a 'Covid' propaganda hotspot at one point. *Discovery Salud*, a

health and medicine magazine, quoted government figures which showed how 17,000 *fewer* people died in Spain in 2020 than in 2019 and more than 26,000 fewer than in 2018. The age-standardised mortality rate for England and Wales when age distribution is taken into account was significantly lower in 2020 than the 1970s, 80s and 90s, and was only the ninth highest since 2000. Where is the 'pandemic'?

Post mortems and autopsies virtually disappeared for 'Covid' deaths amid claims that 'virus-infected' bodily fluids posed a risk to those carrying out the autopsy. This was rejected by renowned German pathologist and forensic doctor Klaus Püschel who said that he and his staff had by then done 150 autopsies on 'Covid' patients with no problems at all. He said they were needed to know why some 'Covid' patients suffered blood clots and not severe respiratory infections. The 'virus' is, after all, called SARS or 'severe acute respiratory syndrome'. I highlighted in the spring of 2020 this phenomenon and quoted New York intensive care doctor Cameron Kyle-Sidell who posted a soon deleted YouTube video to say that they had been told to prepare to treat an infectious disease called 'Covid-19', but that was not what they were dealing with. Instead he likened the lung condition of the most severely ill patients to what you would expect with cabin depressurisation in a plane at 30,000 feet or someone dropped on the top of Everest without oxygen or acclimatisation. I have never said this is not happening to a small minority of alleged 'Covid' patients – I am saying this is not caused by a phantom 'contagious virus'. Indeed Kyle-Sidell said that 'Covid-19' was not the disease they were told was coming their way. 'We are operating under a medical paradigm that is untrue,' he said, and he believed they were treating the wrong disease: 'These people are being slowly starved of oxygen.' Patients would take off their oxygen masks in a state of fear and stress and while they were blue in the face on the brink of death. They did not look like patients dying of pneumonia. You can see why they don't want autopsies when their virus doesn't exist and there is another condition in some people that they don't wish to be uncovered. I should add here that

the 5G system of millimetre waves was being rapidly introduced around the world in 2020 and even more so now as they fire 5G at the Earth from satellites. At 60 gigahertz within the 5G range that frequency interacts with the oxygen molecule and stops people breathing in sufficient oxygen to be absorbed into the bloodstream. They are installing 5G in schools and hospitals. The world is not mad or anything. 5G can cause major changes to the lungs and blood as I detail in *The Answer* and these consequences are labelled 'Covid-19', the alleged symptoms of which can be caused by 5G and other electromagnetic frequencies as cells respond to radiation poisoning.

The 'Covid death' scam

Dr Scott Jensen, a Minnesota state senator and medical doctor, exposed 'Covid' Medicare payment incentives to hospitals and death certificate manipulation. He said he was sent a seven-page document by the US Department of Health 'coaching' him on how to fill out death certificates which had never happened before. The document said that he didn't need to have a laboratory test for 'Covid-19' to put that on the death certificate and that shocked him when death certificates are supposed to be about facts. Jensen described how doctors had been 'encouraged, if not pressured' to make a diagnosis of 'Covid-19' if they thought it was probable or *'presumed*'. No positive test was necessary – not that this would have mattered anyway. He said doctors were told to diagnose 'Covid' by symptoms when these were the same as colds, allergies, other respiratory problems, and certainly with influenza which 'disappeared' in the 'Covid' era. A common sniffle was enough to get the dreaded verdict. Ontario authorities decreed that a single care home resident with *one* symptom from a long list must lead to the isolation of the entire home. Other courageous doctors like Jensen made the same point about death figure manipulation and how deaths by other causes were falling while 'Covid-19 deaths' were rising at the same rate due to re-diagnosis. Their videos rarely survive long on YouTube with its Cult-supporting algorithms courtesy of CEO Susan Wojcicki and her bosses at Google. Figure-tampering was so glaring

and ubiquitous that even officials were letting it slip or outright saying it. UK chief scientific adviser Patrick Vallance said on one occasion that 'Covid' on the death certificate doesn't mean 'Covid' was the cause of death (so why the hell is it there?) and we had the rare sight of a BBC reporter telling the truth when she said: 'Someone could be successfully treated for Covid, in say April, discharged, and then in June, get run over by a bus and die … That person would still be counted as a Covid death in England.' Yet the BBC and the rest of the world media went on repeating the case and death figures as if they were real. Illinois Public Health Director Dr Ngozi Ezike revealed the deceit while her bosses must have been clenching their buttocks:

> If you were in a hospice and given a few weeks to live and you were then found to have Covid that would be counted as a Covid death. [There might be] a clear alternate cause, but it is still listed as a Covid death. So everyone listed as a Covid death doesn't mean that was the cause of the death, but that they had Covid at the time of death.

Yes, a 'Covid virus' never shown to exist and tested for with a test not testing for the 'virus'. In the first period of the pandemic hoax through the spring of 2020 the process began of designating almost everything a 'Covid' death and this has continued ever since. I sat in a restaurant one night listening to a loud conversation on the next table where a family was discussing in bewilderment how a relative who had no symptoms of 'Covid', and had died of a long-term problem, could have been diagnosed a death by the 'virus'. I could understand their bewilderment. If they read this book they will know why this medical fraud has been perpetrated the world over.

Some media truth shock

The media ignored the evidence of death certificate fraud until eventually one columnist did speak out when she saw it first-hand. Bel Mooney is a long-time national newspaper journalist in Britain currently working for the *Daily Mail*. Her article on February 19th, 2021, carried this headline: 'My dad Ted passed three Covid tests

and died of a chronic illness yet he's officially one of Britain's 120,000 victims of the virus and is far from alone ... so how many more are there?' She told how her 99-year-old father was in a care home with a long-standing chronic obstructive pulmonary disease and vascular dementia. Maybe, but he was still aware enough to tell her from the start that there was no 'virus' and he refused the 'vaccine' for that reason. His death was not unexpected given his chronic health problems and Mooney said she was shocked to find that 'Covid-19' was declared the cause of death on his death certificate. She said this was a 'bizarre and unacceptable untruth' for a man with long-time health problems who had tested negative twice at the home for the 'virus'. I was also shocked by this story although not by what she said. I had been highlighting the death certificate manipulation for ten months. It was the confirmation that a professional full-time journalist only realised this was going on when it affected her directly and neither did she know that whether her dad tested positive or negative was irrelevant with the test not testing for the 'virus'. Where had she been? She said she did not believe in 'conspiracy theories' without knowing I'm sure that this and 'conspiracy theorists' were terms put into widespread circulation by the CIA in the 1960s to discredit those who did not accept the ridiculous official story of the Kennedy assassination. A blanket statement of 'I don't believe in conspiracy theories' is always bizarre. The dictionary definition of the term alone means the world is drowning in conspiracies. What she said was even more daft when her dad had just been affected by the 'Covid' conspiracy. Why else does she think that 'Covid-19' was going on the death certificates of people who died of something else?

To be fair once she saw from personal experience what was happening she didn't mince words. Mooney was called by the care home on the morning of February 9th to be told her father had died in his sleep. When she asked for the official cause of death what came back was 'Covid-19'. Mooney challenged this and was told there had been deaths from Covid on the dementia floor (confirmed by a test not testing for the 'virus') so they considered it 'reasonable

to assume'. 'But doctor,' Mooney rightly protested, 'an assumption isn't a diagnosis.' She said she didn't blame the perfectly decent and sympathetic doctor – 'he was just doing his job'. Sorry, but that's *bullshit*. He wasn't doing his job at all. He was putting a false cause of death on the death certificate and that is a criminal offence for which he should be brought to account and the same with the millions of doctors worldwide who have done the same. They were not doing their job they were following orders and that must not wash at new Nuremberg trials any more than it did at the first ones. Mooney's doctor was 'assuming' (presuming) as he was told to, but 'just following orders' makes no difference to his actions. A doctor's job is to serve the patient and the truth, not follow orders, but that's what they have done all over the world and played a central part in making the 'Covid' hoax possible with all its catastrophic consequences for humanity. Shame on them and they must answer for their actions. Mooney said her disquiet worsened when she registered her father's death by telephone and was told by the registrar there had been very many other cases like hers where 'the deceased' had not tested positive for 'Covid' yet it was recorded as the cause of death. The test may not matter, but those involved at their level *think* it matters and it shows a callous disregard for accurate diagnosis. The pressure to do this is coming from the top of the national 'health' pyramids which in turn obey the World Health Organization which obeys Gates and the Cult. Mooney said the registrar agreed that this must distort the national figures adding that 'the strangest thing is that every winter we record countless deaths from flu, and this winter there have been none. Not one!' She asked if the registrar thought deaths from flu were being misdiagnosed and lumped together with 'Covid' deaths. The answer was a 'puzzled yes'. Mooney said that the funeral director said the same about 'Covid' deaths which had nothing to do with 'Covid'. They had lost count of the number of families upset by this and other funeral companies in different countries have had the same experience. Mooney wrote:

The nightly shroud-waving and shocking close-ups of pain imposed on us by the TV news bewildered and terrified the population into eager compliance with lockdowns. We were invited to 'save the NHS' and to grieve for strangers – the real-life loved ones behind those shocking death counts. Why would the public imagine what I now fear, namely that the way Covid-19 death statistics are compiled might make the numbers seem greater than they are?

Oh, just a little bit – like 100 percent.

Do the maths

Mooney asked why a country would wish to skew its mortality figures by wrongly certifying deaths? What had been going on? Well, if you don't believe in conspiracies you will never find the answer which is that *it's a conspiracy*. She did, however, describe what she had discovered as a 'national scandal'. In reality it's a global scandal and happening everywhere. Pillars of this conspiracy were all put into place before the button was pressed with the Drosten PCR protocol and high amplifications to produce the cases and death certificate changes to secure illusory 'Covid' deaths. Mooney notes that normally two doctors were needed to certify a death, with one having to know the patient, and how the rules were changed in the spring of 2020 to allow one doctor to do this. In the same period 'Covid deaths' were decreed to be all cases where Covid-19 was put on the death certificate even without a positive test or any symptoms. Mooney asked: 'How many of the 30,851 (as of January 15) care home resident deaths with Covid-19 on the certificate (32.4 per cent of all deaths so far) were based on an assumption, like that of my father? And what has that done to our national psyche?'All of them is the answer to the first question and it has devastated and dismantled the national psyche, actually the global psyche, on a colossal scale. In the UK case and death data is compiled by organisations like Public Health England (PHE) and the Office for National Statistics (ONS). Mooney highlights the insane policy of counting a death from any cause as 'Covid-19' if this happens within 28 days of a positive test (with a test not testing for the 'virus') and she points out that ONS statistics reflect deaths 'involving Covid' 'or due to Covid' which meant in practice any

death where 'Covid-19' was mentioned on the death certificate. She described the consequences of this fraud:

Most people will accept the narrative they are fed, so panicky governments here and in Europe witnessed the harsh measures enacted in totalitarian China and jumped into lockdown. Headlines about Covid deaths tolled like the knell that would bring doomsday to us all. Fear stalked our empty streets. Politicians parroted the frankly ridiculous aim of 'zero Covid' and shut down the economy, while most British people agreed that lockdown was essential and (astonishingly to me, as a patriotic Brit) even wanted more restrictions.

For what? Lies on death certificates? Never mind the grim toll of lives ruined, suicides, schools closed, rising inequality, depression, cancelled hospital treatments, cancer patients in a torture of waiting, poverty, economic devastation, loneliness, families kept apart, and so on. How many lives have been lost as a direct result of lockdown?

She said that we could join in a national chorus of shock and horror at reaching the 120,000 death toll which was surely certain to have been totally skewed all along, but what about the human cost of lockdown justified by these 'death figures'? *The British Medical Journal* had reported a 1,493 percent increase in cases of children taken to Great Ormond Street Hospital with abusive head injuries alone and then there was the effect on families:

Perhaps the most shocking thing about all this is that families have been kept apart – and obeyed the most irrational, changing rules at the whim of government – because they believed in the statistics. They succumbed to fear, which his generation rejected in that war fought for freedom. Dad (God rest his soul) would be angry. And so am I.

Another theme to watch is that in the winter months when there are more deaths from all causes they focus on 'Covid' deaths and in the summer when the British Lung Foundation says respiratory disease plummets by 80 percent they rage on about 'cases'. Either way fascism on population is always the answer.

Nazi eugenics in the 21st century

Elderly people in care homes have been isolated from their families month after lonely month with no contact with relatives and grandchildren who were banned from seeing them. We were told

that lockdown fascism was to 'protect the vulnerable' like elderly people. At the same time Do Not Resuscitate (DNR) orders were placed on their medical files so that if they needed resuscitation it wasn't done and 'Covid-19' went on their death certificates. Old people were not being 'protected' they were being culled – murdered in truth. DNR orders were being decreed for disabled and young people with learning difficulties or psychological problems. The UK Care Quality Commission, a non-departmental body of the Department of Health and Social Care, found that 34 percent of those working in health and social care were pressured into placing 'do not attempt cardiopulmonary resuscitation' orders on 'Covid' patients who suffered from disabilities and learning difficulties without involving the patient or their families in the decision. UK judges ruled that an elderly woman with dementia should have the DNA-manipulating 'Covid vaccine' against her son's wishes and that a man with severe learning difficulties should have the jab despite his family's objections. Never mind that many had already died. The judiciary always supports doctors and government in fascist dictatorships. They wouldn't dare do otherwise. A horrific video was posted showing fascist officers from Los Angeles police forcibly giving the 'Covid' shot to women with special needs who were screaming that they didn't want it. The same fascists are seen giving the jab to a sleeping elderly woman in a care home. This is straight out of the Nazi playbook. Hitler's Nazis committed mass murder of the mentally ill and physically disabled throughout Germany and occupied territories in the programme that became known as Aktion T4, or just T4. Sabbatian-controlled Hitler and his grotesque crazies set out to kill those they considered useless and unnecessary. The Reich Committee for the Scientific Registering of Hereditary and Congenital Illnesses registered the births of babies identified by physicians to have 'defects'. By 1941 alone more than 5,000 children were murdered by the state and it is estimated that in total the number of innocent people killed in Aktion T4 was between 275,000 and 300,000. Parents were told their children had been sent away for 'special treatment' never to return. It is rather pathetic to see claims about plans for new extermination camps being dismissed today

when the same force behind current events did precisely that 80 years ago. Margaret Sanger was a Cult operative who used 'birth control' to sanitise her programme of eugenics. Organisations she founded became what is now Planned Parenthood. Sanger proposed that 'the whole dysgenic population would have its choice of segregation or sterilization'. These included epileptics, 'feeble-minded', and prostitutes. Sanger opposed charity because it perpetuated 'human waste'. She reveals the Cult mentality and if anyone thinks that extermination camps are a 'conspiracy theory' their naivety is touching if breathtakingly stupid.

If you don't believe that doctors can act with callous disregard for their patients it is worth considering that doctors and medical staff agreed to put government-decreed DNR orders on medical files and do nothing when resuscitation is called for. I don't know what you call such people in your house. In mine they are Nazis from the Josef Mengele School of Medicine. Phenomenal numbers of old people have died worldwide from the effects of lockdown, depression, lack of treatment, the 'vaccine' (more later) and losing the will to live. A common response at the start of the manufactured pandemic was to remove old people from hospital beds and transfer them to nursing homes. The decision would result in a mass cull of elderly people in those homes through lack of treatment – *not* 'Covid'. Care home whistleblowers have told how once the 'Covid' era began doctors would not come to their homes to treat patients and they were begging for drugs like antibiotics that often never came. The most infamous example was ordered by New York governor Andrew Cuomo, brother of a moronic CNN host, who amazingly was given an Emmy Award for his handling of the 'Covid crisis' by the ridiculous Wokers that hand them out. Just how ridiculous could be seen in February, 2021, when a Department of Justice and FBI investigation began into how thousands of old people in New York died in nursing homes after being discharged from hospital to make way for 'Covid' patients on Cuomo's say-so – and how he and his staff covered up these facts. This couldn't have happened to a nicer psychopath. Even then there was a 'Covid' spin. Reports said that

thousands of old people who tested positive for 'Covid' in hospital were transferred to nursing homes to both die of 'Covid' and transmit it to others. No – they were in hospital because they were ill and the fact that they tested positive with a test not testing for the 'virus' is irrelevant. They were ill often with respiratory diseases ubiquitous in old people near the end of their lives. Their transfer out of hospital meant that their treatment stopped and many would go on to die.

They're old. Who gives a damn?

I have exposed in the books for decades the Cult plan to cull the world's old people and even to introduce at some point what they call a 'demise pill' which at a certain age everyone would take and be out of here by law. In March, 2021, Spain legalised euthanasia and assisted suicide following the Netherlands, Belgium, Luxembourg and Canada on the Tiptoe to the demise pill. Treatment of old people by many 'care' homes has been a disgrace in the 'Covid' era. There are many, many, caring staff – I know some. There have, however, been legions of stories about callous treatment of old people and their families. Police were called when families came to take their loved ones home in the light of isolation that was killing them. They became prisoners of the state. Care home residents in insane, fascist Ontario, Canada, were not allowed to leave their *room* once the 'Covid' hoax began. UK staff have even wheeled elderly people away from windows where family members were talking with them. Oriana Criscuolo from Stockport in the English North West dropped off some things for her 80-year-old father who has Parkinson's disease and dementia and she wanted to wave to him through a ground-floor window. She was told that was 'illegal'. When she went anyway they closed the curtains in the middle of the day. Oriana said:

> It's just unbelievable. I cannot understand how care home staff – people who are being paid to care – have become so uncaring. Their behaviour is inhumane and cruel. It's beyond belief.

She was right and this was not a one-off. What a way to end your life in such loveless circumstances. UK registered nurse Nicky Millen, a proper old school nurse for 40 years, said that when she started her career care was based on dignity, choice, compassion and empathy. Now she said 'the things that are important to me have gone out of the window.' She was appalled that people were dying without their loved ones and saying goodbye on iPads. Nicky described how a distressed 89-year-old lady stroked her face and asked her 'how many paracetamol would it take to finish me off'. Life was no longer worth living while not seeing her family. Nicky said she was humiliated in front of the ward staff and patients for letting the lady stroke her face and giving her a cuddle. Such is the dehumanisation that the 'Covid' hoax has brought to the surface. Nicky worked in care homes where patients told her they were being held prisoner. 'I want to live until I die', one said to her. 'I had a lady in tears because she hadn't seen her great-grandson.' Nicky was compassionate old school meeting psychopathic New Normal. She also said she had worked on a 'Covid' ward with no 'Covid' patients. Jewish writer Shai Held wrote an article in March, 2020, which was headlined 'The Staggering, Heartless Cruelty Toward the Elderly'. What he described was happening from the earliest days of lockdown. He said 'the elderly' were considered a group and not unique individuals (the way of the Woke). Shai Held said:

Notice how the all-too-familiar rhetoric of dehumanization works: 'The elderly' are bunched together as a faceless mass, all of them considered culprits and thus effectively deserving of the suffering the pandemic will inflict upon them. Lost entirely is the fact that the elderly are individual human beings, each with a distinctive face and voice, each with hopes and dreams, memories and regrets, friendships and marriages, loves lost and loves sustained.

'The elderly' have become another dehumanised group for which anything goes and for many that has resulted in cold disregard for their rights and their life. The distinctive face that Held talks about is designed to be deleted by masks until everyone is part of a faceless mass.

‘War-zone’ hospitals myth

Again and again medical professionals have told me what was really going on and how hospitals ‘overrun like war zones’ according to the media were virtually empty. The mantra from medical whistleblowers was please don’t use my name or my career is over. Citizen journalists around the world sneaked into hospitals to film evidence exposing the ‘war-zone’ lie. They really *were* largely empty with closed wards and operating theatres. I met a hospital worker in my town on the Isle of Wight during the first lockdown in 2020 who said the only island hospital had never been so quiet. Lockdown was justified by the psychopaths to stop hospitals being overrun. At the same time that the island hospital was near-empty the military arrived here to provide *extra beds*. It was all propaganda to ramp up the fear to ensure compliance with fascism as were never-used temporary hospitals with thousands of beds known as Nightingales and never-used make-shift mortuaries opened by the criminal UK government. A man who helped to install those extra island beds attributed to the army said they were never used and the hospital was empty. Doctors and nurses ‘stood around talking or on their phones, wandering down to us to see what we were doing’. There were no masks or social distancing. He accused the useless local island paper, the *County Press*, of ‘pumping the fear as if our hospital was overrun and we only have one so it should have been’. He described ambulances parked up with crews outside in deck chairs. When his brother called an ambulance he was told there was a two-hour backlog which he called ‘bullshit’. An old lady on the island fell ‘and was in a bad way’, but a caller who rang for an ambulance was told the situation wasn’t urgent enough. Ambulance stations were working under capacity while people would hear ambulances with sirens blaring driving through the streets. When those living near the stations realised what was going on they would follow them as they left, circulated around an urban area with the sirens going, and then came back without stopping. All this was to increase levels of fear and the same goes for the ‘ventilator shortage crisis’ that cost tens of millions for hastily produced ventilators never to be used.

Ambulance crews that agreed to be exploited in this way for fear propaganda might find themselves a mirror. I wish them well with that. Empty hospitals were the obvious consequence of treatment and diagnoses of non-'Covid' conditions cancelled and those involved handed a death sentence. People have been dying at home from undiagnosed and untreated cancer, heart disease and other life-threatening conditions to allow empty hospitals to deal with a 'pandemic' that wasn't happening.

Death of the innocent

'War-zones' have been laying off nursing staff, even doctors where they can. There was no work for them. Lockdown was justified by saving lives and protecting the vulnerable they were actually killing with DNR orders and preventing empty hospitals being 'overrun'. In Britain the mantra of stay at home to 'save the NHS' was everywhere and across the world the same story was being sold when it was all lies. Two California doctors, Dan Erickson and Artin Massihi at Accelerated Urgent Care in Bakersfield, held a news conference in April, 2020, to say that intensive care units in California were 'empty, essentially', with hospitals shutting floors, not treating patients and laying off doctors. The California health system was working at minimum capacity 'getting rid of doctors because we just don't have the volume'. They said that people with conditions such as heart disease and cancer were not coming to hospital out of fear of 'Covid-19'. Their video was deleted by Susan Wojcicki's Cult-owned YouTube after reaching five million views. Florida governor Ron Desantis, who rejected the severe lockdowns of other states and is being targeted for doing so, said that in March, 2020, every US governor was given models claiming they would run out of hospital beds in days. That was never going to happen and the 'modellers' knew it. Deceit can be found at every level of the system. Urgent children's operations were cancelled including fracture repairs and biopsies to spot cancer. Eric Nicholls, a consultant paediatrician, said 'this is obviously concerning and we need to return to normal operating and to increase capacity as soon as possible'. Psychopaths

in power were rather less concerned *because* they are psychopaths. Deletion of urgent care and diagnosis has been happening all over the world and how many kids and others have died as a result of the actions of these cold and heartless lunatics dictating 'health' policy? The number must be stratospheric. Richard Sullivan, professor of cancer and global health at King's College London, said people feared 'Covid' more than cancer such was the campaign of fear. 'Years of lost life will be quite dramatic', Sullivan said, with 'a huge amount of avoidable mortality'. Sarah Woolnough, executive director for policy at Cancer Research UK, said there had been a 75 percent drop in urgent referrals to hospitals by family doctors of people with suspected cancer. Sullivan said that 'a lot of services have had to scale back – we've seen a dramatic decrease in the amount of elective cancer surgery'. Lockdown deaths worldwide has been absolutely fantastic with the *New York Post* reporting how data confirmed that 'lockdowns end more lives than they save':

> There was a sharp decline in visits to emergency rooms and an increase in fatal heart attacks because patients didn't receive prompt treatment. Many fewer people were screened for cancer. Social isolation contributed to excess deaths from dementia and Alzheimer's.
>
> Researchers predicted that the social and economic upheaval would lead to tens of thousands of "deaths of despair" from drug overdoses, alcoholism and suicide. As unemployment surged and mental-health and substance-abuse treatment programs were interrupted, the reported levels of anxiety, depression and suicidal thoughts increased dramatically, as did alcohol sales and fatal drug overdoses.

This has been happening while nurses and other staff had so much time on their hands in the 'war-zones' that Tic-Tok dancing videos began appearing across the Internet with medical staff dancing around in empty wards and corridors as people died at home from causes that would normally have been treated in hospital.

Mentions in dispatches

One brave and truth-committed whistleblower was Louise Hampton, a call handler with the UK NHS who made a viral Internet video saying she had done 'fuck all' during the 'pandemic'

which was 'a load of bollocks'. She said that 'Covid-19' was rebranded flu and of course she lost her job. This is what happens in the medical and endless other professions now when you tell the truth. Louise filmed inside 'war-zone' accident and emergency departments to show they were empty and I mean *empty* as in no one there. The mainstream media could have done the same and blown the gaff on the whole conspiracy. They haven't to their eternal shame. Not that most 'journalists' seem capable of manifesting shame as with the psychopaths they slavishly repeat without question. The relative few who were admitted with serious health problems were left to die alone with no loved ones allowed to see them because of 'Covid' rules and they included kids dying without the comfort of mum and dad at their bedside while the evil behind this couldn't give a damn. It was all good fun to them. A Scottish NHS staff nurse publicly quit in the spring of 2021 saying: 'I can no longer be part of the lies and the corruption by the government.' She said hospitals 'aren't full, the beds aren't full, beds have been shut, wards have been shut'. Hospitals were never busy throughout 'Covid'. The staff nurse said that Nicola Sturgeon, tragically the leader of the Scottish government, was on television saying save the hospitals and the NHS – 'but the beds are empty' and 'we've not seen flu, we always see flu every year'. She wrote to government and spoke with her union Unison (the unions are Cult-compromised and *useless*, but nothing changed. Many of her colleagues were scared of losing their jobs if they spoke out as they wanted to. She said nursing staff were being affected by wearing masks all day and 'my head is splitting every shift from wearing a mask'. The NHS is part of the fascist tyranny and must be dismantled so we can start again with human beings in charge. (Ironically, hospitals were reported to be busier again when official 'Covid' cases *fell* in spring/summer of 2021 and many other conditions required treatment at the same time as *the fake vaccine rollout*.)

I will cover the 'Covid vaccine' scam in detail later, but it is another indicator of the sickening disregard for human life that I am highlighting here. The DNA-manipulating concoctions do not fulfil

the definition of a 'vaccine', have never been used on humans before and were given only emergency approval because trials were not completed and they continued using the unknowing public. The result was what a NHS senior nurse with responsibility for 'vaccine' procedure said was 'genocide'. She said the 'vaccines' were not 'vaccines'. They had not been shown to be safe and claims about their effectiveness by drug companies were 'poetic licence'. She described what was happening as a 'horrid act of human annihilation'. The nurse said that management had instigated a policy of not providing a Patient Information Leaflet (PIL) before people were 'vaccinated' even though health care professionals are supposed to do this according to protocol. Patients should also be told that they are taking part in an ongoing clinical trial. Her challenges to what is happening had seen her excluded from meetings and ridiculed in others. She said she was told to 'watch my step … or I would find myself surplus to requirements'. The nurse, who spoke anonymously in fear of her career, said she asked her NHS manager why he/she was content with taking part in genocide against those having the 'vaccines'. The reply was that everyone had to play their part and to 'put up, shut up, and get it done'. Government was 'leaning heavily' on NHS management which was clearly leaning heavily on staff. This is how the global 'medical' hierarchy operates and it starts with the Cult and its World Health Organization.

She told the story of a doctor who had the Pfizer jab and when questioned had no idea what was in it. The doctor had never read the literature. We have to stop treating doctors as intellectual giants when so many are moral and medical pygmies. The doctor did not even know that the 'vaccines' were not fully approved or that their trials were ongoing. They were, however, asking their patients if they minded taking part in follow-ups for research purposes – yes, the *ongoing clinical trial*. The nurse said the doctor's ignorance was not rare and she had spoken to a hospital consultant who had the jab without any idea of the background or that the 'trials' had not been completed. Nurses and pharmacists had shown the same ignorance.

'My NHS colleagues have forsaken their duty of care, broken their code of conduct – Hippocratic Oath – and have been brainwashed just the same as the majority of the UK public through propaganda …' She said she had not been able to recruit a single NHS colleague, doctor, nurse or pharmacist to stand with her and speak out. Her union had refused to help. She said that if the genocide came to light she would not hesitate to give evidence at a Nuremberg-type trial against those in power who could have affected the outcomes but didn't.

And all for what?

To put the nonsense into perspective let's say the 'virus' does exist and let's go completely crazy and accept that the official manipulated figures for cases and deaths are accurate. *Even then* a study by Stanford University epidemiologist Dr John Ioannidis published on the World Health Organization website produced an average infection to fatality rate of … *0.23 percent!* Ioannidis said: 'If one could sample equally from all locations globally, the median infection fatality rate might even be substantially lower than the 0.23% observed in my analysis.' For healthy people under 70 it was … *0.05 percent*! This compares with the 3.4 percent claimed by the Cult-owned World Health Organization when the hoax was first played and maximum fear needed to be generated. An updated Stanford study in April, 2021, put the 'infection' to 'fatality' rate at just 0.15 percent. Another team of scientists led by Megan O'Driscoll and Henrik Salje studied data from 45 countries and published their findings on the Nature website. For children and young people the figure is so small it virtually does not register although authorities will be hyping dangers to the young when they introduce DNA-manipulating 'vaccines' for children. The O'Driscoll study produced an average infection-fatality figure of 0.003 for children from birth to four; 0.001 for 5 to 14; 0.003 for 15 to 19; and it was still only 0.456 up to 64. To claim that children must be 'vaccinated' to protect them from 'Covid' is an obvious lie and so there must be another reason and there is. What's more the average age of a 'Covid' death is akin

to the average age that people die in general. The average age of death in England is about 80 for men and 83 for women. The average age of death from alleged 'Covid' is between 82 and 83. California doctors, Dan Erickson and Artin Massihi, said at their April media conference that projection models of millions of deaths had been 'woefully inaccurate'. They produced detailed figures showing that Californians had a 0.03 chance of dying from 'Covid' based on the number of people who tested positive (with a test not testing for the 'virus'). Erickson said there was a 0.1 percent chance of dying from 'Covid' in the *state* of New York, not just the city, and a 0.05 percent chance in Spain, a centre of 'Covid-19' hysteria at one stage. The Stanford studies supported the doctors' data with fatality rate estimates of 0.23 and 0.15 percent. How close are these figures to my estimate of *zero*? Death-rate figures claimed by the World Health Organization at the start of the hoax were some 15 times higher. The California doctors said there was no justification for lockdowns and the economic devastation they caused. Everything they had ever learned about quarantine was that you quarantine the *sick* and not the healthy. They had never seen this before and it made no medical sense.

Why in the in the light of all this would governments and medical systems the world over say that billions must go under house arrest; lose their livelihood; in many cases lose their mind, their health and their life; force people to wear masks dangerous to health and psychology; make human interaction and even family interaction a criminal offence; ban travel; close restaurants, bars, watching live sport, concerts, theatre, and any activity involving human togetherness and discourse; and closing schools to isolate children from their friends and cause many to commit suicide in acts of hopelessness and despair? The California doctors said lockdown consequences included increased child abuse, partner abuse, alcoholism, depression, and other impacts they were seeing every day. Who would do that to the entire human race if not mentally-ill psychopaths of almost unimaginable extremes like Bill Gates? We must face the reality of what we are dealing with and come out of

denial. Fascism and tyranny are made possible only by the target population submitting and acquiescing to fascism and tyranny. The whole of human history shows that to be true. Most people naively and unquestioning believed what they were told about a 'deadly virus' and meekly and weakly submitted to house arrest. Those who didn't believe it – at least in total – still submitted in fear of the consequences of not doing so. For the rest who wouldn't submit draconian fines have been imposed, brutal policing by psychopaths *for* psychopaths, and condemnation from the meek and weak who condemn the Pushbackers on behalf of the very force that has them, too, in its gunsights. 'Pathetic' does not even begin to suffice. Britain's brainless 'Health' Secretary Matt Hancock warned anyone lying to border officials about returning from a list of 'hotspot' countries could face a jail sentence of up to ten years which is more than for racially-aggravated assault, incest and attempting to have sex with a child under 13. Hancock is a lunatic, but he has the state apparatus behind him in a Cult-led chain reaction and the same with UK 'Vaccine Minister' Nadhim Zahawi, a prominent member of the mega-Cult secret society, Le Cercle, which featured in my earlier books. The Cult enforces its will on governments and medical systems; government and medical systems enforce their will on business and police; business enforces its will on staff who enforce it on customers; police enforce the will of the Cult on the population and play their essential part in creating a world of fascist control that their own children and grandchildren will have to live in their entire lives. It is a hierarchical pyramid of imposition and acquiescence and, yes indeedy, of clinical insanity.

Does anyone bright enough to read this book have to ask what the answer is? I think not, but I will reveal it anyway in the fewest of syllables: Tell the psychos and their moronic lackeys to fuck off and let's get on with our lives. We are many – They are few.

CHAPTER SEVEN

War on your mind

One believes things because one has been conditioned to believe them
Aldous Huxley, *Brave New World*

I have described the 'Covid' hoax as a 'Psyop' and that is true in every sense and on every level in accordance with the definition of that term which is psychological warfare. Break down the 'Covid pandemic' to the foundation themes and it is psychological warfare on the human individual and collective mind.

The same can be said for the entire human belief system involving every subject you can imagine. Huxley was right in his contention that people believe what they are conditioned to believe and this comes from the repetition throughout their lives of the same falsehoods. They spew from government, corporations, media and endless streams of 'experts' telling you what the Cult wants you to believe and often believing it themselves (although *far* from always). 'Experts' are rewarded with 'prestigious' jobs and titles and as agents of perceptual programming with regular access to the media. The Cult has to control the narrative – control *information* – or they lose control of the vital, crucial, without-which-they-cannot-prevail public perception of reality. The foundation of that control today is the Internet made possible by the Defense Advanced Research Projects Agency (DARPA), the incredibly sinister technological arm of the Pentagon. The Internet is the result of military technology.

DARPA openly brags about establishing the Internet which has been a long-term project to lasso the minds of the global population. I have said for decades the plan is to control information to such an extreme that eventually no one would see or hear anything that the Cult does not approve. We are closing in on that end with ferocious censorship since the 'Covid' hoax began and in my case it started back in the 1990s in terms of books and speaking venues. I had to create my own publishing company in 1995 precisely because no one else would publish my books even then. I think they're all still running.

Cult Internet

To secure total control of information they needed the Internet in which pre-programmed algorithms can seek out 'unclean' content for deletion and even stop it being posted in the first place. The Cult had to dismantle print and non-Internet broadcast media to ensure the transfer of information to the appropriate-named 'Web' – a critical expression of the *Cult* web. We've seen the ever-quickening demise of traditional media and control of what is left by a tiny number of corporations operating worldwide. Independent journalism in the mainstream is already dead and never was that more obvious than since the turn of 2020. The Cult wants all information communicated via the Internet to globally censor and allow the plug to be pulled any time. Lockdowns and forced isolation has meant that communication between people has been through electronic means and no longer through face-to-face discourse and discussion. Cult psychopaths have targeted the bars, restaurants, sport, venues and meeting places in general for this reason. None of this is by chance and it's to stop people gathering in any kind of privacy or number while being able to track and monitor all Internet communications and block them as necessary. Even private messages between individuals have been censored by these fascists that control Cult fronts like Facebook, Twitter, Google and YouTube which are all officially run by Sabbatian place-people and from the background by higher-level Sabbatian place people.

Facebook, Google, Amazon and their like were seed-funded and supported into existence with money-no-object infusions of funds either directly or indirectly from DARPA and CIA technology arm In-Q-Tel. The Cult plays the long game and prepares very carefully for big plays like 'Covid'. Amazon is another front in the psychological war and pretty much controls the global market in book sales and increasingly publishing. Amazon's limitless funds have deleted fantastic numbers of independent publishers to seize global domination on the way to deciding which books can be sold and circulated and which cannot. Moves in that direction are already happening. Amazon's leading light Jeff Bezos is the grandson of Lawrence Preston Gise who worked with DARPA predecessor ARPA. Amazon has big connections to the CIA and the Pentagon. The plan I have long described went like this:

1. Employ military technology to establish the Internet.

2. Sell the Internet as a place where people can freely communicate without censorship and allow that to happen until the Net becomes the central and irreversible pillar of human society. If the Internet had been highly censored from the start many would have rejected it.

3. Fund and manipulate major corporations into being to control the circulation of information on your Internet using cover stories about geeks in garages to explain how they came about. Give them unlimited funds to expand rapidly with no need to make a profit for years while non-Cult companies who need to balance the books cannot compete. You know that in these circumstances your Googles, YouTubes, Facebooks and Amazons are going to secure near monopolies by either crushing or buying up the opposition.

4. Allow freedom of expression on both the Internet and communication platforms to draw people in until the Internet is the central and irreversible pillar of human society and your communication corporations have reached a stage of near monopoly domination.

5. Then unleash your always-planned frenzy of censorship on the basis of 'where else are you going to go?' and continue to expand that until nothing remains that the Cult does not want its human targets to see.

The process was timed to hit the 'Covid' hoax to ensure the best chance possible of controlling the narrative which they knew they had to do at all costs. They were, after all, about to unleash a 'deadly virus' that didn't really exist. If you do that in an environment of free-flowing information and opinion you would be dead in the

water before you could say Gates is a psychopath. The network was in place through which the Cult-created-and-owned World Health Organization could dictate the 'Covid' narrative and response policy slavishly supported by Cult-owned Internet communication giants and mainstream media while those telling a different story were censored. Google, YouTube, Facebook and Twitter openly announced that they would do this. What else would we expect from Cult-owned operations like Facebook which former executives have confirmed set out to make the platform more addictive than cigarettes and coldly manipulates emotions of its users to sow division between people and groups and scramble the minds of the young? If Zuckerberg lives out the rest of his life without going to jail for crimes against humanity, and most emphatically against the young, it will be a travesty of justice. Still, no matter, cause and effect will catch up with him eventually and the same with Sergey Brin and Larry Page at Google with its CEO Sundar Pichai who fix the Google search results to promote Cult narratives and hide the opposition. Put the same key words into Google and other search engines like DuckDuckGo and you will see how different results can be. Wikipedia is another intensely biased 'encyclopaedia' which skews its content to the Cult agenda. YouTube links to Wikipedia's version of 'Covid' and 'climate change' on video pages in which experts in their field offer a different opinion (even that is increasingly rare with Wojcicki censorship). Into this 'Covid' silence-them network must be added government media censors, sorry 'regulators', such as Ofcom in the UK which imposed tyrannical restrictions on British broadcasters that had the effect of banning me from ever appearing. Just to debate with me about my evidence and views on 'Covid' would mean breaking the fascistic impositions of Ofcom and its CEO career government bureaucrat Melanie Dawes. Gutless British broadcasters tremble at the very thought of fascist Ofcom.

Psychos behind 'Covid'

The reason for the 'Covid' catastrophe in all its facets and forms can be seen by whom and what is driving the policies worldwide in such a coordinated way. Decisions are not being made to protect health, but to target psychology. The dominant group guiding and 'advising' government policy are not medical professionals. They are psychologists and behavioural scientists. Every major country has its own version of this phenomenon and I'll use the British example to show how it works. In many ways the British version has been affecting the wider world in the form of the huge behaviour manipulation network in the UK which operates in other countries. The network involves private companies, government, intelligence and military. The Cabinet Office is at the centre of the government 'Covid' Psyop and part-owns, with 'innovation charity' Nesta, the Behavioural Insights Team (BIT) which claims to be independent of government but patently isn't. The BIT was established in 2010 and its job is to manipulate the psyche of the population to acquiesce to government demands and so much more. It is also known as the 'Nudge Unit', a name inspired by the 2009 book by two ultra-Zionists, Cass Sunstein and Richard Thaler, called *Nudge: Improving Decisions About Health, Wealth, and Happiness*. The book, as with the Behavioural Insights Team, seeks to 'nudge' behaviour (manipulate it) to make the public follow patterns of action and perception that suit those in authority (the Cult). Sunstein is so skilled at this that he advises the World Health Organization and the UK Behavioural Insights Team and was Administrator of the White House Office of Information and Regulatory Affairs in the Obama administration. Biden appointed him to the Department of Homeland Security – another ultra-Zionist in the fold to oversee new immigration laws which is another policy the Cult wants to control. Sunstein is desperate to silence anyone exposing conspiracies and co-authored a 2008 report on the subject in which suggestions were offered to ban 'conspiracy theorizing' or impose 'some kind of tax, financial or otherwise, on those who disseminate such theories'. I guess a psychiatrist's chair is out of the question?

Sunstein's mate Richard Thaler, an 'academic affiliate' of the UK Behavioural Insights Team, is a proponent of 'behavioural economics' which is defined as the study of 'the effects of psychological, cognitive, emotional, cultural and social factors on the decisions of individuals and institutions'. Study the effects so they can be manipulated to be what you want them to be. Other leading names in the development of behavioural economics are ultra-Zionists Daniel Kahneman and Robert J. Shiller and they, with Thaler, won the Nobel Memorial Prize in Economic Sciences for their work in this field. The Behavioural Insights Team is operating at the heart of the UK government and has expanded globally through partnerships with several universities including Harvard, Oxford, Cambridge, University College London (UCL) and Pennsylvania. They claim to have 'trained' (reframed) 20,000 civil servants and run more than 750 projects involving 400 randomised controlled trials in dozens of countries' as another version of mind reframers Common Purpose. BIT works from its office in New York with cities and their agencies, as well as other partners, across the United States and Canada – this is a company part-owned by the British government Cabinet Office. An executive order by President Cult-servant Obama established a US Social and Behavioral Sciences Team in 2015. They all have the same reason for being and that's to brainwash the population directly and by brainwashing those in positions of authority.

'Covid' mind game

Another prime aspect of the UK mind-control network is the 'independent' [joke] Scientific Pandemic Insights Group on Behaviours (SPI-B) which 'provides behavioural science advice aimed at anticipating and helping people adhere to interventions that are recommended by medical or epidemiological experts'. That means manipulating public perception and behaviour to do whatever government tells them to do. It's disgusting and if they really want the public to be 'safe' this lot should all be under lock and key. According to the government website SPI-B consists of

'behavioural scientists, health and social psychologists, anthropologists and historians' and advises the Whitty-Vallance-led Scientific Advisory Group for Emergencies (SAGE) which in turn advises the government on 'the science' (it doesn't) and 'Covid' policy. When politicians say they are being guided by 'the science' this is the rabble in each country they are talking about and that 'science' is dominated by behaviour manipulators to enforce government fascism through public compliance. The Behaviour Insight Team is headed by psychologist David Solomon Halpern, a visiting professor at King's College London, and connects with a national and global web of other civilian and military organisations as the Cult moves towards its goal of fusing them into one fascistic whole in every country through its 'Fusion Doctrine'. The behaviour manipulation network involves, but is not confined to, the Foreign Office; National Security Council; government communications headquarters (GCHQ); MI5; MI6; the Cabinet Office-based Media Monitoring Unit; and the Rapid Response Unit which 'monitors digital trends to spot emerging issues; including misinformation and disinformation; and identifies the best way to respond'.

There is also the 77th Brigade of the UK military which operates like the notorious Israeli military's Unit 8200 in manipulating information and discussion on the Internet by posing as members of the public to promote the narrative and discredit those who challenge it. Here we have the military seeking to manipulate *domestic* public opinion while the Nazis in government are fine with that. Conservative Member of Parliament Tobias Ellwood, an advocate of lockdown and control through 'vaccine passports', is a Lieutenant Colonel reservist in the 77th Brigade which connects with the military operation jHub, the 'innovation centre' for the Ministry of Defence and Strategic Command. jHub has also been involved with the civilian National Health Service (NHS) in 'symptom tracing' the population. The NHS is a key part of this mind control network and produced a document in December, 2020, explaining to staff how to use psychological manipulation with different groups and ages to get them to have the DNA-manipulating 'Covid vaccine'

that's designed to cumulatively rewrite human genetics. The document, called 'Optimising Vaccination Roll Out – Do's and Dont's for all messaging, documents and "communications" in the widest sense', was published by NHS England and the NHS Improvement *Behaviour Change Unit* in partnership with Public Health England and Warwick Business School. I hear the mantra about 'save the NHS' and 'protect the NHS' when we need to scrap the NHS and start again. The current version is far too corrupt, far too anti-human and totally compromised by Cult operatives and their assets. UK government broadcast media censor Ofcom will connect into this web – as will the BBC with its tremendous Ofcom influence – to control what the public see and hear and dictate mass perception. Nuremberg trials must include personnel from all these organisations.

The fear factor

The 'Covid' hoax has led to the creation of the UK Cabinet Office-connected Joint Biosecurity Centre (JBC) which is officially described as providing 'expert advice on pandemics' using its independent [all Cult operations are 'independent'] analytical function to provide real-time analysis about infection outbreaks to identify and respond to outbreaks of Covid-19'. Another role is to advise the government on a response to spikes in infections – 'for example by closing schools or workplaces in local areas where infection levels have risen'. Put another way, promoting the Cult agenda. The Joint Biosecurity Centre is modelled on the Joint Terrorism Analysis Centre which analyses intelligence to set 'terrorism threat levels' and here again you see the fusion of civilian and military operations and intelligence that has led to military intelligence producing documents about 'vaccine hesitancy' and how it can be combated. Domestic civilian matters and opinions should not be the business of the military. The Joint Biosecurity Centre is headed by Tom Hurd, director general of the Office for Security and Counter-Terrorism from the establishment-to-its-fingertips Hurd family. His father is former Foreign Secretary Douglas Hurd. How coincidental that Tom

Hurd went to the elite Eton College and Oxford University with Boris Johnson. Imperial College with its ridiculous computer modeller Neil Ferguson will connect with this gigantic web that will itself interconnect with similar set-ups in other major and not so major countries. Compared with this Cult network the politicians, be they Boris Johnson, Donald Trump or Joe Biden, are bit-part players 'following the science'. The network of psychologists was on the 'Covid' case from the start with the aim of generating maximum fear of the 'virus' to ensure compliance by the population. A government behavioural science group known as SPI-B produced a paper in March, 2020, for discussion by the main government science advisory group known as SAGE. It was headed 'Options for increasing adherence to social distancing measures' and it said the following in a section headed 'Persuasion':

- A substantial number of people still do not feel sufficiently personally threatened; it could be that they are reassured by the low death rate in their demographic group, although levels of concern may be rising. Having a good understanding of the risk has been found to be positively associated with adoption of COVID-19 social distancing measures in Hong Kong.

- The perceived level of personal threat needs to be increased among those who are complacent, using hard-hitting evaluation of options for increasing social distancing emotional messaging. To be effective this must also empower people by making clear the actions they can take to reduce the threat.

- Responsibility to others: There seems to be insufficient understanding of, or feelings of responsibility about, people's role in transmitting the infection to others … Messaging about actions need to be framed positively in terms of protecting oneself and the community, and increase confidence that they will be effective.

- Some people will be more persuaded by appeals to play by the rules, some by duty to the community, and some to personal risk.

> All these different approaches are needed. The messaging also needs to take account of the realities of different people's lives. Messaging needs to take account of the different motivational levers and circumstances of different people.

All this could be achieved the SPI-B psychologists said by *using the media to increase the sense of personal threat* which translates as terrify the shit out of the population, including children, so they all do what we want. That's not happened has it? Those excuses for 'journalists' who wouldn't know journalism if it bit them on the arse (the great majority) have played their crucial part in serving this Cult-government Psyop to enslave their own kids and grandkids. How they live with themselves I have no idea. The psychological war has been underpinned by constant government 'Covid' propaganda in almost every television and radio ad break, plus the Internet and print media, which has pounded out the fear with taxpayers footing the bill for their own programming. The result has been people terrified of a 'virus' that doesn't exist or one with a tiny fatality rate even if you believe it does. People walk down the street and around the shops wearing face-nappies damaging their health and psychology while others report those who refuse to be that naïve to the police who turn up in their own face-nappies. I had a cameraman come to my flat and he was so frightened of 'Covid' he came in wearing a mask and refused to shake my hand in case he caught something. He had – naïveitis – and the thought that he worked in the mainstream media was both depressing and made his behaviour perfectly explainable. The fear which has gripped the minds of so many and frozen them into compliance has been carefully cultivated by these psychologists who are really psychopaths. If lives get destroyed and a lot of young people commit suicide it shows our plan is working. SPI-B then turned to compulsion on the public to comply. 'With adequate preparation, rapid change can be achieved', it said. Some countries had introduced mandatory self-isolation on a wide scale without evidence of major public unrest and a large majority of the UK's population appeared to be supportive of more coercive measures with 64 percent of adults saying they would

support putting London under a lockdown (watch the 'polls' which are designed to make people believe that public opinion is in favour or against whatever the subject in hand).

For 'aggressive protective measures' to be effective, the SPI-B paper said, special attention should be devoted to those population groups that are more at risk. Translated from the Orwellian this means making the rest of population feel guilty for not protecting the 'vulnerable' such as old people which the Cult and its agencies were about to kill on an industrial scale with lockdown, lack of treatment and the Gates 'vaccine'. Psychopath psychologists sold their guilt-trip so comprehensively that Los Angeles County Supervisor Hilda Solis reported that children were apologising (from a distance) to their parents and grandparents for bringing 'Covid' into their homes and getting them sick. '… These apologies are just some of the last words that loved ones will ever hear as they die alone,' she said. Gut-wrenchingly Solis then used this childhood tragedy to tell children to stay at home and 'keep your loved ones alive'. Imagine heaping such potentially life-long guilt on a kid when it has absolutely nothing to do with them. These people are deeply disturbed and the psychologists behind this even more so.

Uncivil war – divide and rule

Professional mind-controllers at SPI-B wanted the media to increase a sense of responsibility to others (do as you're told) and promote 'positive messaging' for those actions while in contrast to invoke 'social disapproval' by the unquestioning, obedient, community of anyone with a mind of their own. Again the compliant Goebbels-like media obliged. This is an old, old, trick employed by tyrannies the world over throughout human history. You get the target population to keep the target population in line – *your* line. SPI-B said this could 'play an important role in preventing anti-social behaviour or discouraging failure to enact pro-social behaviour'. For 'anti-social' in the Orwellian parlance of SPI-B see any behaviour that government doesn't approve. SPI-B recommendations said that 'social disapproval' should be accompanied by clear messaging and

promotion of strong collective identity – hence the government and celebrity mantra of 'we're all in this together'. Sure we are. The mind doctors have such contempt for their targets that they think some clueless comedian, actor or singer telling them to do what the government wants will be enough to win them over. We have had UK comedian Lenny Henry, actor Michael Caine and singer Elton John wheeled out to serve the propagandists by urging people to have the DNA-manipulating 'Covid' non-'vaccine'. The role of Henry and fellow black celebrities in seeking to coax a 'vaccine' reluctant black community into doing the government's will was especially stomach-turning. An emotion-manipulating script and carefully edited video featuring these black 'celebs' was such an insult to the intelligence of black people and where's the self-respect of those involved selling their souls to a fascist government agenda? Henry said he heard black people's 'legitimate worries and concerns', but people must 'trust the facts' when they were doing exactly that by not having the 'vaccine'. They had to include the obligatory reference to Black Lives Matter with the line … 'Don't let coronavirus cost even more black lives – because we matter'. My god, it was pathetic. 'I know the vaccine is safe and what it does.' How? 'I'm a comedian and it says so in my script.'

SPI-B said social disapproval needed to be carefully managed to avoid victimisation, scapegoating and misdirected criticism, but they knew that their 'recommendations' would lead to exactly that and the media were specifically used to stir-up the divide-and-conquer hostility. Those who conform like good little baa, baas, are praised while those who have seen through the tidal wave of lies are 'Covidiots'. The awake have been abused by the fast asleep for not conforming to fascism and impositions that the awake know are designed to endanger their health, dehumanise them, and tear asunder the very fabric of human society. We have had the curtain-twitchers and morons reporting neighbours and others to the face-nappied police for breaking 'Covid rules' with fascist police delighting in posting links and phone numbers where this could be done. The Cult cannot impose its will without a compliant police

and military or a compliant population willing to play their part in enslaving themselves and their kids. The words of a pastor in Nazi Germany are so appropriate today:

First they came for the socialists and I did not speak out because I was not a socialist.

Then they came for the trade unionists and I did not speak out because I was not a trade unionist.

Then they came for the Jews and I did not speak out because I was not a Jew.

Then they came for me and there was no one left to speak for me.

Those who don't learn from history are destined to repeat it and so many are.

'Covid' rules: Rewiring the mind

With the background laid out to this gigantic national and global web of psychological manipulation we can put 'Covid' rules into a clear and sinister perspective. Forget the claims about protecting health. 'Covid' rules are about dismantling the human mind, breaking the human spirit, destroying self-respect, and then putting Humpty Dumpty together again as a servile, submissive slave. Social isolation through lockdown and distancing have devastating effects on the human psyche as the psychological psychopaths well know and that's the real reason for them. Humans need contact with each other, discourse, closeness and touch, or they eventually, and literarily, go crazy. Masks, which I will address at some length, fundamentally add to the effects of isolation and the Cult agenda to dehumanise and de-individualise the population. To do this while knowing – in fact *seeking* – this outcome is the very epitome of evil and psychologists involved in this *are* the epitome of evil. They must like all the rest of the Cult demons and their assets stand trial for crimes against humanity on a scale that defies the imagination. Psychopaths in uniform use isolation to break enemy troops and agents and make them subservient and submissive to tell what they know. The technique is rightly considered a form of torture and

torture is most certainly what has been imposed on the human population.

Clinically-insane American psychologist Harry Harlow became famous for his isolation experiments in the 1950s in which he separated baby monkeys from their mothers and imprisoned them for months on end in a metal container or 'pit of despair'. They soon began to show mental distress and depression as any idiot could have predicted. Harlow put other monkeys in steel chambers for three, six or twelve months while denying them any contact with animals or humans. He said that the effects of total social isolation for six months were 'so devastating and debilitating that we had assumed initially that twelve months of isolation would not produce any additional decrement'; but twelve months of isolation 'almost obliterated the animals socially'. This is what the Cult and its psychopaths are doing to you and your children. Even monkeys in partial isolation in which they were not allowed to form relationships with other monkeys became 'aggressive and hostile, not only to others, but also towards their own bodies'. We have seen this in the young as a consequence of lockdown. UK government psychopaths launched a public relations campaign telling people not to hug each other even after they received the 'Covid-19 vaccine' which we were told with more lies would allow a return to 'normal life'. A government source told *The Telegraph*: 'It will be along the lines that it is great that you have been vaccinated, but if you are going to visit your family and hug your grandchildren there is a chance you are going to infect people you love.' The source was apparently speaking from a secure psychiatric facility. Janet Lord, director of Birmingham University's Institute of Inflammation and Ageing, said that parents and grandparents should avoid hugging their children. Well, how can I put it, Ms Lord? Fuck off. Yep, that'll do.

Destroying the kids – where are the parents?

Observe what has happened to people enslaved and isolated by lockdown as suicide and self-harm has soared worldwide,

particularly among the young denied the freedom to associate with their friends. A study of 49,000 people in English-speaking countries concluded that almost half of young adults are at clinical risk of mental health disorders. A national survey in America of 1,000 currently enrolled high school and college students found that 5 percent reported attempting suicide during the pandemic. Data from the US CDC's National Syndromic Surveillance Program from January 1st to October 17th, 2020, revealed a *31 percent* increase in mental health issues among adolescents aged 12 to 17 compared with 2019. The CDC reported that America in general suffered the biggest drop in life expectancy since World War Two as it fell by a year in the first half of 2020 as a result of 'deaths of despair' – overdoses and suicides. Deaths of despair have leapt by more than 20 percent during lockdown and include the highest number of fatal overdoses ever recorded in a single year – 81,000. Internet addiction is another consequence of being isolated at home which lowers interest in physical activities as kids fall into inertia and what's the point? Children and young people are losing hope and giving up on life, sometimes literally. A 14-year-old boy killed himself in Maryland because he had 'given up' when his school district didn't reopen; an 11-year-old boy shot himself during a zoom class; a teenager in Maine succumbed to the isolation of the 'pandemic' when he ended his life after experiencing a disrupted senior year at school. Children as young as nine have taken their life and all these stories can be repeated around the world. Careers are being destroyed before they start and that includes those in sport in which promising youngsters have not been able to take part. The plan of the psycho-psychologists is working all right. Researchers at Cambridge University found that lockdowns cause significant harm to children's mental health. Their study was published in the *Archives of Disease in Childhood*, and followed 168 children aged between 7 and 11. The researchers concluded:

> During the UK lockdown, children's depression symptoms have increased substantially, relative to before lockdown. The scale of this effect has direct relevance for the continuation of different elements of lockdown policy, such as complete or partial school closures …

… Specifically, we observed a statistically significant increase in ratings of depression, with a medium-to-large effect size. Our findings emphasise the need to incorporate the potential impact of lockdown on child mental health in planning the ongoing response to the global pandemic and the recovery from it.

Not a chance when the Cult's psycho-psychologists were getting exactly what they wanted. The UK's Royal College of Paediatrics and Child Health has urged parents to look for signs of eating disorders in children and young people after a three to four fold increase. Specialists say the 'pandemic' is a major reason behind the rise. You don't say. The College said isolation from friends during school closures, exam cancellations, loss of extra-curricular activities like sport, and an increased use of social media were all contributory factors along with fears about the virus (psycho-psychologists again), family finances, and students being forced to quarantine. Doctors said young people were becoming severely ill by the time they were seen with 'Covid' regulations reducing face-to-face consultations. Nor is it only the young that have been devastated by the psychopaths. Like all bullies and cowards the Cult is targeting the young, elderly, weak and infirm. A typical story was told by a British lady called Lynn Parker who was not allowed to visit her husband in 2020 for the last ten and half months of his life 'when he needed me most' between March 20th and when he died on December 19th. This vacates the criminal and enters the territory of evil. The emotional impact on the immune system alone is immense as are the number of people of all ages worldwide who have died as a result of Cult-demanded, Gates-demanded, lockdowns.

Isolation is torture

The experience of imposing solitary confinement on millions of prisoners around the world has shown how a large percentage become 'actively psychotic and/or acutely suicidal'. Social isolation has been found to trigger 'a specific psychiatric syndrome, characterized by hallucinations; panic attacks; overt paranoia; diminished impulse control; hypersensitivity to external stimuli; and difficulties with thinking, concentration and memory'. Juan Mendez,

a United Nations rapporteur (investigator), said that isolation is a form of torture. Research has shown that even after isolation prisoners find it far more difficult to make social connections and I remember chatting to a shop assistant after one lockdown who told me that when her young son met another child again he had no idea how to act or what to do. Hannah Flanagan, Director of Emergency Services at Journey Mental Health Center in Dane County, Wisconsin, said: 'The specificity about Covid social distancing and isolation that we've come across as contributing factors to the suicides are really new to us this year.' But they are not new to those that devised them. They are getting the effect they want as the population is psychologically dismantled to be rebuilt in a totally different way. Children and the young are particularly targeted. They will be the adults when the full-on fascist AI-controlled technocracy is planned to be imposed and they are being prepared to meekly submit. At the same time older people who still have a memory of what life was like before – and how fascist the new normal really is – are being deleted. You are going to see efforts to turn the young against the old to support this geriatric genocide. Hannah Flanagan said the big increase in suicide in her county proved that social isolation is not only harmful, but deadly. Studies have shown that isolation from others is one of the main risk factors in suicide and even more so with women. Warnings that lockdown could create a 'perfect storm' for suicide were ignored. After all this was one of the *reasons* for lockdown. Suicide, however, is only the most extreme of isolation consequences. There are many others. Dr Dhruv Khullar, assistant professor of healthcare policy at Weill Cornell Medical College, said in a *New York Times* article in 2016 long before the fake 'pandemic':

> A wave of new research suggests social separation is bad for us. Individuals with less social connection have disrupted sleep patterns, altered immune systems, more inflammation and higher levels of stress hormones. One recent study found that isolation increases the risk of heart disease by 29 percent and stroke by 32 percent. Another analysis that pooled data from 70 studies and 3.4 million people found that socially isolated individuals had a 30 percent higher risk of dying in the next seven years, and that this effect was largest in middle age.

Loneliness can accelerate cognitive decline in older adults, and isolated individuals are twice as likely to die prematurely as those with more robust social interactions. These effects start early: Socially isolated children have significantly poorer health 20 years later, even after controlling for other factors. All told, loneliness is as important a risk factor for early death as obesity and smoking.

There you have proof from that one article alone four years before 2020 that those who have enforced lockdown, social distancing and isolation knew what the effect would be and that is even more so with professional psychologists that have been driving the policy across the globe. We can go back even further to the years 2000 and 2003 and the start of a major study on the effects of isolation on health by Dr Janine Gronewold and Professor Dirk M. Hermann at the University Hospital in Essen, Germany, who analysed data on 4,316 people with an average age of 59 who were recruited for the long-term research project. They found that socially isolated people are more than 40 percent more likely to have a heart attack, stroke, or other major cardiovascular event and nearly 50 percent more likely to die from any cause. Given the financial Armageddon unleashed by lockdown we should note that the study found a relationship between increased cardiovascular risk and lack of financial support. After excluding other factors social isolation was still connected to a 44 percent increased risk of cardiovascular problems and a 47 percent increased risk of death by any cause. Lack of financial support was associated with a 30 percent increase in the risk of cardiovascular health events. Dr Gronewold said it had been known for some time that feeling lonely or lacking contact with close friends and family can have an impact on physical health and the study had shown that having strong social relationships is of high importance for heart health. Gronewold said they didn't understand yet why people who are socially isolated have such poor health outcomes, but this was obviously a worrying finding, particularly during these times of prolonged social distancing. Well, it can be explained on many levels. You only have to identify the point in the body where people feel loneliness and missing people they are parted from – it's in the centre of the chest where they feel the ache of loneliness and the ache of missing people. 'My heart aches for

you' … 'My heart aches for some company.' I will explain this more in the chapter Escaping Wetiko, but when you realise that the body is the mind – they are expressions of each other – the reason why state of the mind dictates state of the body becomes clear.

American psychologist Ranjit Powar was highlighting the effects of lockdown isolation as early as April, 2020. She said humans have evolved to be social creatures and are wired to live in interactive groups. Being isolated from family, friends and colleagues could be unbalancing and traumatic for most people and could result in short or even long-term psychological and physical health problems. An increase in levels of anxiety, aggression, depression, forgetfulness and hallucinations were possible psychological effects of isolation. 'Mental conditions may be precipitated for those with underlying pre-existing susceptibilities and show up in many others without any pre-condition.' Powar said personal relationships helped us cope with stress and if we lost this outlet for letting off steam the result can be a big emotional void which, for an average person, was difficult to deal with. 'Just a few days of isolation can cause increased levels of anxiety and depression' – so what the hell has been the effect on the global population of *18 months* of this at the time of writing? Powar said: 'Add to it the looming threat of a dreadful disease being repeatedly hammered in through the media and you have a recipe for many shades of mental and physical distress.' For those with a house and a garden it is easy to forget that billions have had to endure lockdown isolation in tiny overcrowded flats and apartments with nowhere to go outside. The psychological and physical consequences of this are unimaginable and with lunatic and abusive partners and parents the consequences have led to tremendous increases in domestic and child abuse and alcoholism as people seek to shut out the horror. Ranjit Powar said:

Staying in a confined space with family is not all a rosy picture for everyone. It can be extremely oppressive and claustrophobic for large low-income families huddled together in small single-room houses. Children here are not lucky enough to have many board/electronic games or books to keep them occupied.

Add to it the deep insecurity of running out of funds for food and basic necessities. On the other hand, there are people with dysfunctional family dynamics, such as domineering, abusive or alcoholic partners, siblings or parents which makes staying home a period of trial. Incidence of suicide and physical abuse against women has shown a worldwide increase. Heightened anxiety and depression also affect a person's immune system, making them more susceptible to illness.

To think that Powar's article was published on April 11th, 2020.

Six-feet fantasy

Social (unsocial) distancing demanded that people stay six feet or two metres apart. UK government advisor Robert Dingwall from the New and Emerging Respiratory Virus Threats Advisory Group said in a radio interview that the two-metre rule was 'conjured up out of nowhere' and was not based on science. No, it was not based on *medical* science, but it didn't come out of nowhere. The distance related to *psychological* science. Six feet/two metres was adopted in many countries and we were told by people like the criminal Anthony Fauci and his ilk that it was founded on science. Many schools could not reopen because they did not have the space for six-feet distancing. Then in March, 2021, after a year of six-feet 'science', a study published in the *Journal of Infectious Diseases* involving more than 500,000 students and almost 100,000 staff over 16 weeks revealed no significant difference in 'Covid' cases between six feet and three feet and Fauci changed his tune. Now three feet was okay. There is no difference between six feet and three *inches* when there is no 'virus' and they got away with six feet for psychological reasons for as long as they could. I hear journalists and others talk about 'unintended consequences' of lockdown. They are not *unintended* at all; they have been coldly-calculated for a specific outcome of human control and that's why super-psychopaths like Gates have called for them so vehemently. Super-psychopath psychologists have demanded them and psychopathic or clueless, spineless, politicians have gone along with them by 'following the science'. But it's not science at all. 'Science' is not what is; it's only what people can be manipulated to believe it is. The whole 'Covid' catastrophe is

founded on mind control. Three word or three statement mantras issued by the UK government are a well-known mind control technique and so we've had 'Stay home/protect the NHS/save lives', 'Stay alert/control the virus/save lives' and 'hands/face/space'. One of the most vocal proponents of extreme 'Covid' rules in the UK has been Professor Susan Michie, a member of the British Communist Party, who is not a medical professional. Michie is the director of the Centre for Behaviour Change at University College London. She is a *behavioural psychologist* and another filthy rich 'Marxist' who praised China's draconian lockdown. She was known by fellow students at Oxford University as 'Stalin's nanny' for her extreme Marxism. Michie is an influential member of the UK government's Scientific Advisory Group for Emergencies (SAGE) and behavioural manipulation groups which have dominated 'Covid' policy. She is a consultant adviser to the World Health Organization on 'Covid-19' and behaviour. Why the hell are lockdowns anything to do with her when they are claimed to be about health? Why does a behavioural psychologist from a group charged with changing the behaviour of the public want lockdown, human isolation and mandatory masks? Does that question really need an answer? Michie *absolutely* has to explain herself before a Nuremberg court when humanity takes back its world again and even more so when you see the consequences of masks that she demands are compulsory. This is a Michie classic:

> The benefits of getting primary school children to wear masks is that regardless of what little degree of transmission is occurring in those age groups it could help normalise the practice. Young children wearing masks may be more likely to get their families to accept masks.

Those words alone should carry a prison sentence when you ponder on the callous disregard for children involved and what a statement it makes about the mind and motivations of Susan Michie. What a lovely lady and what she said there encapsulates the mentality of the psychopaths behind the 'Covid' horror. Let us compare what Michie said with a countrywide study in Germany published at researchsquare.com involving 25,000 school children and 17,854 health complaints submitted by parents. Researchers

found that masks are harming children physically, psychologically, and behaviourally with 24 health issues associated with mask wearing. They include: shortness of breath (29.7%); dizziness (26.4%); increased headaches (53%); difficulty concentrating (50%); drowsiness or fatigue (37%); and malaise (42%). Nearly a third of children experienced more sleep issues than before and a quarter developed new fears. Researchers found health issues and other impairments in 68 percent of masked children covering their faces for an average of 4.5 hours a day. Hundreds of those taking part experienced accelerated respiration, tightness in the chest, weakness, and short-term impairment of consciousness. A reminder of what Michie said again:

The benefits of getting primary school children to wear masks is that regardless of what little degree of transmission is occurring in those age groups it could help normalise the practice. Young children wearing masks may be more likely to get their families to accept masks.

Psychopaths in government and psychology now have children and young people – plus all the adults – wearing masks for hours on end while clueless teachers impose the will of the psychopaths on the young they should be protecting. What the hell are parents doing?

Cult lab rats

We have some schools already imposing on students microchipped buzzers that activate when they get 'too close' to their pals in the way they do with lab rats. How apt. To the Cult and its brain-dead servants our children *are* lab rats being conditioned to be unquestioning, dehumanised slaves for the rest of their lives. Children and young people are being weaned and frightened away from the most natural human instincts including closeness and touch. I have tracked in the books over the years how schools were banning pupils from greeting each other with a hug and the whole Cult-induced Me Too movement has terrified men and boys from a relaxed and natural interaction with female friends and work colleagues to the point where many men try never to be in a room

alone with a woman that's not their partner. Airhead celebrities have as always played their virtue-signalling part in making this happen with their gross exaggeration. For every monster like Harvey Weinstein there are at least tens of thousands of men that don't treat women like that; but everyone must be branded the same and policy changed for them as well as the monster. I am going to be using the word 'dehumanise' many times in this chapter because that is what the Cult is seeking to do and it goes very deep as we shall see. Don't let them kid you that social distancing is planned to end one day. That's not the idea. We are seeing more governments and companies funding and producing wearable gadgets to keep people apart and they would not be doing that if this was meant to be short-term. A tech start-up company backed by GCHQ, the British Intelligence and military surveillance headquarters, has created a social distancing wrist sensor that alerts people when they get too close to others. The CIA has also supported tech companies developing similar devices. The wearable sensor was developed by Tended, one of a number of start-up companies supported by GCHQ (see the CIA and DARPA). The device can be worn on the wrist or as a tag on the waistband and will vibrate whenever someone wearing the device breaches social distancing and gets anywhere near natural human contact. The company had a lucky break in that it was developing a distancing sensor when the 'Covid' hoax arrived which immediately provided a potentially enormous market. How fortunate. The government in big-time Cult-controlled Ontario in Canada is investing $2.5 million in wearable contact tracing technology that 'will alert users if they may have been exposed to the Covid-19 in the workplace and will beep or vibrate if they are within six feet of another person'. Facedrive Inc., the technology company behind this, was founded in 2016 with funding from the Ontario Together Fund and obviously they, too, had a prophet on the board of directors. The human surveillance and control technology is called TraceSCAN and would be worn by the human cyborgs in places such as airports, workplaces, construction sites, care homes and … *schools*.

I emphasise schools with children and young people the prime targets. You know what is planned for society as a whole if you keep your eyes on the schools. They have always been places where the state program the next generation of slaves to be its compliant worker-ants – or Woker-ants these days; but in the mist of the 'Covid' madness they have been transformed into mind laboratories on a scale never seen before. Teachers and head teachers are just as programmed as the kids – often more so. Children are kept apart from human interaction by walk lanes, classroom distancing, staggered meal times, masks, and the rolling-out of buzzer systems. Schools are now physically laid out as a laboratory maze for lab-rats. Lunatics at a school in Anchorage, Alaska, who should be prosecuted for child abuse, took away desks and forced children to kneel (know your place) on a mat for five hours a day while wearing a mask and using their chairs as a desk. How this was supposed to impact on a 'virus' only these clinically insane people can tell you and even then it would be clap-trap. The school banned recess (interaction), art classes (creativity), and physical exercise (getting body and mind moving out of inertia). Everyone behind this outrage should be in jail or better still a mental institution. The behavioural manipulators are all for this dystopian approach to schools. Professor Susan Michie, the mind-doctor and British Communist Party member, said it was wrong to say that schools were safe. They had to be made so by 'distancing', masks and ventilation (sitting all day in the cold). I must ask this lady round for dinner on a night I know I am going to be out and not back for weeks. She probably wouldn't be able to make it, anyway, with all the visits to her own psychologist she must have block-booked.

Masking identity

I know how shocking it must be for you that a behaviour manipulator like Michie wants everyone to wear masks which have long been a feature of mind-control programs like the infamous MKUltra in the United States, but, there we are. We live and learn. I spent many years from 1996 to right across the millennium

researching mind control in detail on both sides of the Atlantic and elsewhere. I met a large number of mind-control survivors and many had been held captive in body and mind by MKUltra. MK stands for mind-control, but employs the German spelling in deference to the Nazis spirited out of Germany at the end of World War Two by Operation Paperclip in which the US authorities, with help from the Vatican, transported Nazi mind-controllers and engineers to America to continue their work. Many of them were behind the creation of NASA and they included Nazi scientist and SS officer Wernher von Braun who swapped designing V-2 rockets to bombard London with designing the Saturn V rockets that powered the NASA moon programme's Apollo craft. I think I may have mentioned that the Cult has no borders. Among Paperclip escapees was Josef Mengele, the Angel of Death in the Nazi concentration camps where he conducted mind and genetic experiments on children often using twins to provide a control twin to measure the impact of his 'work' on the other. If you want to observe the Cult mentality in all its extremes of evil then look into the life of Mengele. I have met many people who suffered mercilessly under Mengele in the United States where he operated under the name Dr Greene and became a stalwart of MKUltra programming and torture. Among his locations was the underground facility in the Mojave Desert in California called the China Lake Naval Weapons Station which is almost entirely below the surface. My books *The Biggest Secret*, *Children of the Matrix* and *The Perception Deception* have the detailed background to MKUltra.

The best-known MKUltra survivor is American Cathy O'Brien. I first met her and her late partner Mark Phillips at a conference in Colorado in 1996. Mark helped her escape and deprogram from decades of captivity in an offshoot of MKUltra known as Project Monarch in which 'sex slaves' were provided for the rich and famous including Father George Bush, Dick Cheney and the Clintons. Read Cathy and Mark's book *Trance-Formation of America* and if you are new to this you will be shocked to the core. I read it in 1996 shortly before, with the usual synchronicity of my life, I found

myself given a book table at the conference right next to hers. MKUltra never ended despite being very publicly exposed (only a small part of it) in the 1970s and continues in other guises. I am still in touch with Cathy. She contacted me during 2020 after masks became compulsory in many countries to tell me how they were used as part of MKUltra programming. I had been observing 'Covid regulations' and the relationship between authority and public for months. I saw techniques that I knew were employed on individuals in MKUltra being used on the global population. I had read many books and manuals on mind control including one called *Silent Weapons for Quiet Wars* which came to light in the 1980s and was a guide on how to perceptually program on a mass scale. 'Silent Weapons' refers to mind-control. I remembered a line from the manual as governments, medical authorities and law enforcement agencies have so obviously talked to – or rather at – the adult population since the 'Covid' hoax began as if they are children. The document said:

> If a person is spoken to by a T.V. advertiser as if he were a twelve-year-old, then, due to suggestibility, he will, with a certain probability, respond or react to that suggestion with the uncritical response of a twelve-year-old and will reach in to his economic reservoir and deliver its energy to buy that product on impulse when he passes it in the store.

That's why authority has spoken to adults like children since all this began.

Why did Michael Jackson wear masks?

Every aspect of the 'Covid' narrative has mind-control as its central theme. Cathy O'Brien wrote an article for davidicke.com about the connection between masks and mind control. Her daughter Kelly who I first met in the 1990s was born while Cathy was still held captive in MKUltra. Kelly was forced to wear a mask as part of her programming from the age of *two* to dehumanise her, target her sense of individuality and reduce the amount of oxygen her brain and body received. *Bingo*. This is the real reason for compulsory

masks, why they have been enforced en masse, and why they seek to increase the number they demand you wear. First one, then two, with one disgraceful alleged 'doctor' recommending four which is nothing less than a death sentence. Where and how often they must be worn is being expanded for the purpose of mass mind control and damaging respiratory health which they can call 'Covid-19'. Canada's government headed by the man-child Justin Trudeau, says it's fine for children of two and older to wear masks. An insane 'study' in Italy involving just 47 children concluded there was no problem for babies as young as *four months* wearing them. Even after people were 'vaccinated' they were still told to wear masks by the criminal that is Anthony Fauci. Cathy wrote that mandating masks is allowing the authorities literally to control the air we breathe which is what was done in MKUltra. You might recall how the singer Michael Jackson wore masks and there is a reason for that. He was subjected to MKUltra mind control through Project Monarch and his psyche was scrambled by these simpletons. Cathy wrote:

In MKUltra Project Monarch mind control, Michael Jackson had to wear a mask to silence his voice so he could not reach out for help. Remember how he developed that whisper voice when he wasn't singing? Masks control the mind from the outside in, like the redefining of words is doing. By controlling what we can and cannot say for fear of being labeled racist or beaten, for example, it ultimately controls thought that drives our words and ultimately actions (or lack thereof).

Likewise, a mask muffles our speech so that we are not heard, which controls voice … words … mind. This is Mind Control. Masks are an obvious mind control device, and I am disturbed so many people are complying on a global scale. Masks depersonalize while making a person feel as though they have no voice. It is a barrier to others. People who would never choose to comply but are forced to wear a mask in order to keep their job, and ultimately their family fed, are compromised. They often feel shame and are subdued. People have stopped talking with each other while media controls the narrative.

The 'no voice' theme has often become literal with train passengers told not to speak to each other in case they pass on the 'virus', singing banned for the same reason and bonkers California officials telling people riding roller coasters that they cannot shout and scream. Cathy said she heard every day from healed MKUltra survivors who cannot wear a mask without flashing back on ways

their breathing was controlled – 'from ball gags and penises to water boarding'. She said that through the years when she saw images of people in China wearing masks 'due to pollution' that it was really to control their oxygen levels. 'I knew it was as much of a population control mechanism of depersonalisation as are burkas', she said. Masks are another Chinese communist/fascist method of control that has been swept across the West as the West becomes China at lightning speed since we entered 2020.

Mask-19

There are other reasons for mandatory masks and these include destroying respiratory health to call it 'Covid-19' and stunting brain development of children and the young. Dr Margarite Griesz-Brisson MD, PhD, is a Consultant Neurologist and Neurophysiologist and the Founder and Medical Director of the London Neurology and Pain Clinic. Her CV goes down the street and round the corner. She is clearly someone who cares about people and won't parrot the propaganda. Griesz-Brisson has a PhD in pharmacology, with special interest in neurotoxicology, environmental medicine, neuroregeneration and neuroplasticity (the way the brain can change in the light of information received). She went public in October, 2020, with a passionate warning about the effects of mask-wearing laws:

> The reinhalation of our exhaled air will without a doubt create oxygen deficiency and a flooding of carbon dioxide. We know that the human brain is very sensitive to oxygen deprivation. There are nerve cells for example in the hippocampus that can't be longer than 3 minutes without oxygen – they cannot survive. The acute warning symptoms are headaches, drowsiness, dizziness, issues in concentration, slowing down of reaction time – reactions of the cognitive system.

Oh, I know, let's tell bus, truck and taxi drivers to wear them and people working machinery. How about pilots, doctors and police? Griesz-Brisson makes the important point that while the symptoms she mentions may fade as the body readjusts this does not alter the fact that people continue to operate in oxygen deficit with long list of

potential consequences. She said it was well known that neurodegenerative diseases take years or decades to develop. 'If today you forget your phone number, the breakdown in your brain would have already started 20 or 30 years ago.' She said degenerative processes in your brain are getting amplified as your oxygen deprivation continues through wearing a mask. Nerve cells in the brain are unable to divide themselves normally in these circumstances and lost nerve cells will no longer be regenerated. 'What is gone is gone.' Now consider that people like shop workers and *schoolchildren* are wearing masks for hours every day. What in the name of sanity is going to be happening to them? 'I do not wear a mask, I need my brain to think', Griesz-Brisson said, 'I want to have a clear head when I deal with my patients and not be in a carbon dioxide-induced anaesthesia'. If you are told to wear a mask anywhere ask the organisation, police, store, whatever, for their risk assessment on the dangers and negative effects on mind and body of enforcing mask-wearing. They won't have one because it has never been done not even by government. All of them must be subject to class-action lawsuits as the consequences come to light. They don't do mask risk assessments for an obvious reason. They know what the conclusions would be and independent scientific studies that *have* been done tell a horror story of consequences.

'Masks are criminal'

Dr Griesz-Brisson said that for children and adolescents, masks are an absolute no-no. They had an extremely active and adaptive immune system and their brain was incredibly active with so much to learn. 'The child's brain, or the youth's brain, is thirsting for oxygen.' The more metabolically active an organ was, the more oxygen it required; and in children and adolescents every organ was metabolically active. Griesz-Brisson said that to deprive a child's or adolescent's brain of oxygen, or to restrict it in any way, was not only dangerous to their health, it was absolutely criminal. 'Oxygen deficiency inhibits the development of the brain, and the damage that has taken place as a result CANNOT be reversed.' Mind

manipulators of MKUltra put masks on two-year-olds they wanted to neurologically rewire and you can see why. Griesz-Brisson said a child needs the brain to learn and the brain needs oxygen to function. 'We don't need a clinical study for that. This is simple, indisputable physiology.' Consciously and purposely induced oxygen deficiency was an absolutely deliberate health hazard, and an absolute medical contraindication which means that 'this drug, this therapy, this method or measure should not be used, and is not allowed to be used'. To coerce an entire population to use an absolute medical contraindication by force, she said, there had to be definite and serious reasons and the reasons must be presented to competent interdisciplinary and independent bodies to be verified and authorised. She had this warning of the consequences that were coming if mask wearing continued:

When, in ten years, dementia is going to increase exponentially, and the younger generations couldn't reach their god-given potential, it won't help to say 'we didn't need the masks'. I know how damaging oxygen deprivation is for the brain, cardiologists know how damaging it is for the heart, pulmonologists know how damaging it is for the lungs. Oxygen deprivation damages every single organ. Where are our health departments, our health insurance, our medical associations? It would have been their duty to be vehemently against the lockdown and to stop it and stop it from the very beginning.

Why do the medical boards issue punishments to doctors who give people exemptions? Does the person or the doctor seriously have to prove that oxygen deprivation harms people? What kind of medicine are our doctors and medical associations representing? Who is responsible for this crime? The ones who want to enforce it? The ones who let it happen and play along, or the ones who don't prevent it?

All of the organisations and people she mentions there either answer directly to the Cult or do whatever hierarchical levels above them tell them to do. The outcome of both is the same. 'It's not about masks, it's not about viruses, it's certainly not about your health', Griesz-Brisson said. 'It is about much, much more. I am not participating. I am not afraid.' They were taking our air to breathe and there was no unfounded medical exemption from face masks. Oxygen deprivation was dangerous for every single brain. It had to be the free decision of every human being whether they want to

wear a mask that was absolutely ineffective to protect themselves from a virus. She ended by rightly identifying where the responsibility lies for all this:

> The imperative of the hour is personal responsibility. We are responsible for what we think, not the media. We are responsible for what we do, not our superiors. We are responsible for our health, not the World Health Organization. And we are responsible for what happens in our country, not the government.

Halle-bloody-lujah.

But surgeons wear masks, right?

Independent studies of mask-wearing have produced a long list of reports detailing mental, emotional and physical dangers. What a definition of insanity to see police officers imposing mask-wearing on the public which will cumulatively damage their health while the police themselves wear masks that will cumulatively damage *their* health. It's utter madness and both public and police do this because 'the government says so' – yes a government of brain-donor idiots like UK Health Secretary Matt Hancock reading the 'follow the science' scripts of psychopathic, lunatic psychologists. The response you get from Stockholm syndrome sufferers defending the very authorities that are destroying them and their families is that 'surgeons wear masks'. This is considered the game, set and match that they must work and don't cause oxygen deficit. Well, actually, scientific studies have shown that they *do* and oxygen levels are monitored in operating theatres to compensate. Surgeons wear masks to stop spittle and such like dropping into open wounds – not to stop 'viral particles' which are so miniscule they can only be seen through an electron microscope. Holes in the masks are significantly bigger than 'viral particles' and if you sneeze or cough they will breach the mask. I watched an incredibly disingenuous 'experiment' that claimed to prove that masks work in catching 'virus' material from the mouth and nose. They did this with a slow motion camera and the mask did block big stuff which stayed inside the mask and

against the face to be breathed in or cause infections on the face as we have seen with many children. 'Viral particles', however, would never have been picked up by the camera as they came through the mask when they are far too small to be seen. The 'experiment' was therefore disingenuous *and* useless.

Studies have concluded that wearing masks in operating theatres (and thus elsewhere) make no difference to preventing infection while the opposite is true with toxic shite building up in the mask and this had led to an explosion in tooth decay and gum disease dubbed by dentists 'mask mouth'. You might have seen the Internet video of a furious American doctor urging people to take off their masks after a four-year-old patient had been rushed to hospital the night before and nearly died with a lung infection that doctors sourced to mask wearing. A study in the journal *Cancer Discovery* found that inhalation of harmful microbes can contribute to advanced stage lung cancer in adults and long-term use of masks can help breed dangerous pathogens. Microbiologists have said frequent mask wearing creates a moist environment in which microbes can grow and proliferate before entering the lungs. The Canadian Agency for Drugs and Technologies in Health, or CADTH, a Canadian national organisation that provides research and analysis to healthcare decision-makers, said this as long ago as 2013 in a report entitled 'Use of Surgical Masks in the Operating Room: A Review of the Clinical Effectiveness and Guidelines'. It said:

- No evidence was found to support the use of surgical face masks to reduce the frequency of surgical site infections
- No evidence was found on the effectiveness of wearing surgical face masks to protect staff from infectious material in the operating room.
- Guidelines recommend the use of surgical face masks by staff in the operating room to protect both operating room staff and patients (despite the lack of evidence).

We were told that the world could go back to 'normal' with the arrival of the 'vaccines'. When they came, fraudulent as they are, the story changed as I knew that it would. We are in the midst of transforming 'normal', not going back to it. Mary Ramsay, head of immunisation at Public Health England, echoed the words of US criminal Anthony Fauci who said masks and other regulations must stay no matter if people are vaccinated. The Fauci idiot continued to wear two masks – different colours so both could be clearly seen – after he *claimed* to have been vaccinated. Senator Rand Paul told Fauci in one exchange that his double-masks were 'theatre' and he was right. It's all theatre. Mary Ramsay back-tracked on the vaccine-return-to-normal theme when she said the public may need to wear masks and social-distance for years despite the jabs. 'People have got used to those lower-level restrictions now, and [they] can live with them', she said telling us what the idea has been all along. 'The vaccine does not give you a pass, even if you have had it, you must continue to follow all the guidelines' said a Public Health England statement which reneged on what we had been told before and made having the 'vaccine' irrelevant to 'normality' even by the official story. Spain's fascist government trumped everyone by passing a law mandating the wearing of masks on the beach and even when swimming in the sea. The move would have devastated what's left of the Spanish tourist industry, posed potential breathing dangers to swimmers and had Northern European sunbathers walking around with their forehead brown and the rest of their face white as a sheet. The ruling was so crazy that it had to be retracted after pressure from public and tourist industry, but it confirmed where the Cult wants to go with masks and how clinically insane authority has become. The determination to make masks permanent and hide the serious dangers to body and mind can be seen in the censorship of scientist Professor Denis Rancourt by Bill Gates-funded academic publishing website ResearchGate over his papers exposing the dangers and uselessness of masks. Rancourt said:

ResearchGate today has permanently locked my account, which I have had since 2015. Their reasons graphically show the nature of their attack against democracy, and their corruption of

science … By their obscene non-logic, a scientific review of science articles reporting on harms caused by face masks has a 'potential to cause harm'. No criticism of the psychological device (face masks) is tolerated, if the said criticism shows potential to influence public policy.

This is what happens in a fascist world.

Where are the 'greens' (again)?

Other dangers of wearing masks especially regularly relate to the inhalation of minute plastic fibres into the lungs and the deluge of discarded masks in the environment and oceans. Estimates predicted that more than 1.5 billion disposable masks will end up in the world's oceans every year polluting the water with tons of plastic and endangering marine wildlife. Studies project that humans are using 129 billion face masks each month worldwide – about three million a minute. Most are disposable and made from plastic, non-biodegradable microfibers that break down into smaller plastic particles that become widespread in ecosystems. They are littering cities, clogging sewage channels and turning up in bodies of water. I have written in other books about the immense amounts of microplastics from endless sources now being absorbed into the body. Rolf Halden, director of the Arizona State University (ASU) Biodesign Center for Environmental Health Engineering, was the senior researcher in a 2020 study that analysed 47 human tissue samples and found microplastics in all of them. 'We have detected these chemicals of plastics in every single organ that we have investigated', he said. I wrote in *The Answer* about the world being deluged with microplastics. A study by the Worldwide Fund for Nature (WWF) found that people are consuming on average every week some 2,000 tiny pieces of plastic mostly through water and also through marine life and the air. Every year humans are ingesting enough microplastics to fill a heaped dinner plate and in a life-time of 79 years it is enough to fill two large waste bins. Marco Lambertini, WWF International director general said: 'Not only are plastics polluting our oceans and waterways and killing marine life – it's in all of us and we can't escape consuming plastics,' American

geologists found tiny plastic fibres, beads and shards in rainwater samples collected from the remote slopes of the Rocky Mountain National Park near Denver, Colorado. Their report was headed: 'It is raining plastic.' Rachel Adams, senior lecturer in Biomedical Science at Cardiff Metropolitan University, said that among health consequences are internal inflammation and immune responses to a 'foreign body'. She further pointed out that microplastics become carriers of toxins including mercury, pesticides and dioxins (a known cause of cancer and reproductive and developmental problems). These toxins accumulate in the fatty tissues once they enter the body through microplastics. Now this is being compounded massively by people putting plastic on their face and throwing it away.

Workers exposed to polypropylene plastic fibres known as 'flock' have developed 'flock worker's lung' from inhaling small pieces of the flock fibres which can damage lung tissue, reduce breathing capacity and exacerbate other respiratory problems. *Now* … commonly used surgical masks have three layers of melt-blown textiles made of … polypropylene. We have billions of people putting these microplastics against their mouth, nose and face for hours at a time day after day in the form of masks. How does anyone think that will work out? I mean – what could possibly go wrong? We posted a number of scientific studies on this at davidicke.com, but when I went back to them as I was writing this book the links to the science research website where they were hosted were dead. Anything that challenges the official narrative in any way is either censored or vilified. The official narrative is so unsupportable by the evidence that only deleting the truth can protect it. A study by Chinese scientists still survived – with the usual twist which it why it was still active, I guess. Yes, they found that virtually all the masks they tested increased the daily intake of microplastic fibres, but people should still wear them because the danger from the 'virus' was worse said the crazy 'team' from the Institute of Hydrobiology in Wuhan. Scientists first discovered microplastics in lung tissue of some patients who died of lung cancer

in the 1990s. Subsequent studies have confirmed the potential health damage with the plastic degrading slowly and remaining in the lungs to accumulate in volume. Wuhan researchers used a machine simulating human breathing to establish that masks shed up to nearly 4,000 microplastic fibres in a month with reused masks producing more. Scientists said some masks are laced with toxic chemicals and a variety of compounds seriously restricted for both health and environmental reasons. They include cobalt (used in blue dye) and formaldehyde known to cause watery eyes, burning sensations in the eyes, nose, and throat, plus coughing, wheezing and nausea. No – that must be 'Covid-19'.

Mask 'worms'

There is another and potentially even more sinister content of masks. Mostly new masks of different makes filmed under a microscope around the world have been found to contain strange black fibres or 'worms' that appear to move or 'crawl' by themselves and react to heat and water. The nearest I have seen to them are the self-replicating fibres that are pulled out through the skin of those suffering from Morgellons disease which has been connected to the phenomena of 'chemtrails' which I will bring into the story later on. Morgellons fibres continue to grow outside the body and have a form of artificial intelligence. Black 'worm' fibres in masks have that kind of feel to them and there is a nanotechnology technique called 'worm micelles' which carry and release drugs or anything else you want to deliver to the body. For sure the suppression of humanity by mind altering drugs is the Cult agenda big time and the more excuses they can find to gain access to the body the more opportunities there are to make that happen whether through 'vaccines' or masks pushed against the mouth and nose for hours on end.

So let us summarise the pros and cons of masks:

Against masks: Breathing in your own carbon dioxide; depriving the body and brain of sufficient oxygen; build-up of toxins in the mask that can be breathed into the lungs and cause rashes on the face and 'mask-mouth'; breathing microplastic fibres and toxic chemicals into the lungs; dehumanisation and deleting individualisation by literally making people faceless; destroying human emotional interaction through facial expression and deleting parental connection with their babies which look for guidance to their facial expression.

For masks: They don't protect you from a 'virus' that doesn't exist and even if it did 'viral' particles are so minute they are smaller than the holes in the mask.

Governments, police, supermarkets, businesses, transport companies, and all the rest who seek to impose masks have done no risk assessment on their consequences for health and psychology and are now open to group lawsuits when the impact becomes clear with a cumulative epidemic of respiratory and other disease. Authorities will try to exploit these effects and hide the real cause by dubbing them 'Covid-19'. Can you imagine setting out to force the population to wear health-destroying masks without doing any assessment of the risks? It is criminal and it is evil, but then how many people targeted in this way, who see their children told to wear them all day at school, have asked for a risk assessment? Billions can't be imposed upon by the few unless the billions allow it. Oh, yes, with just a tinge of irony, 85 percent of all masks made worldwide come from *China*.

Wash your hands in toxic shite

'Covid' rules include the use of toxic sanitisers and again the health consequences of constantly applying toxins to be absorbed through the skin is obvious to any level of Renegade Mind. America's Food and Drug Administration (FDA) said that sanitisers are drugs and issued a warning about 75 dangerous brands which contain

methanol used in antifreeze and can cause death, kidney damage and blindness. The FDA circulated the following warning even for those brands that it claims to be safe:

Store hand sanitizer out of the reach of pets and children, and children should use it only with adult supervision. Do not drink hand sanitizer. This is particularly important for young children, especially toddlers, who may be attracted by the pleasant smell or brightly colored bottles of hand sanitizer.

Drinking even a small amount of hand sanitizer can cause alcohol poisoning in children. (However, there is no need to be concerned if your children eat with or lick their hands after using hand sanitizer.) During this coronavirus pandemic, poison control centers have had an increase in calls about accidental ingestion of hand sanitizer, so it is important that adults monitor young children's use.

Do not allow pets to swallow hand sanitizer. If you think your pet has eaten something potentially dangerous, call your veterinarian or a pet poison control center right away. Hand sanitizer is flammable and should be stored away from heat and flames. When using hand sanitizer, rub your hands until they feel completely dry before performing activities that may involve heat, sparks, static electricity, or open flames.

There you go, perfectly safe, then, and that's without even a mention of the toxins absorbed through the skin. Come on kids – sanitise your hands everywhere you go. It will save you from the 'virus'. Put all these elements together of the 'Covid' normal and see how much health and psychology is being cumulatively damaged, even devastated, to 'protect your health'. Makes sense, right? They are only imposing these things because they care, right? *Right?*

Submitting to insanity

Psychological reframing of the population goes very deep and is done in many less obvious ways. I hear people say how contradictory and crazy 'Covid' rules are and how they are ever changing. This is explained away by dismissing those involved as idiots. It is a big mistake. The Cult is delighted if its cold calculation is perceived as incompetence and idiocy when it is anything but. Oh, yes, there are idiots within the system – lots of them – but they are *administering* the Cult agenda, mostly unknowingly. They are not deciding and dictating it. The bulwark against tyranny is self-

respect, always has been, always will be. It is self-respect that has broken every tyranny in history. By its very nature self-respect will not bow to oppression and its perpetrators. There is so little self-respect that it's always the few that overturn dictators. Many may eventually follow, but the few with the iron spines (self-respect) kick it off and generate the momentum. The Cult targets self-respect in the knowledge that once this has gone only submission remains. Crazy, contradictory, ever-changing 'Covid' rules are systematically applied by psychologists to delete self-respect. They *want* you to see that the rules make no sense. It is one thing to decide to do something when *you* have made the choice based on evidence and logic. You still retain your self-respect. It is quite another when you can see what you are being told to do is insane, ridiculous and makes no sense, and *yet you still do it*. Your self-respect is extinguished and this has been happening as ever more obviously stupid and nonsensical things have been demanded and the great majority have complied even when they can see they are stupid and nonsensical.

People walk around in face-nappies knowing they are damaging their health and make no difference to a 'virus'. They do it in fear of not doing it. I know it's daft, but I'll do it anyway. When that happens something dies inside of you and submissive reframing has begun. Next there's a need to hide from yourself that you have conceded your self-respect and you convince yourself that you have not really submitted to fear and intimidation. You begin to believe that you are complying with craziness because it's the right thing to do. When first you concede your self-respect of 2+2 = 4 to 2+2 = 5 you *know* you are compromising your self-respect. Gradually to avoid facing that fact you begin to *believe* that 2+2=5. You have been reframed and I have been watching this process happening in the human psyche on an industrial scale. The Cult is working to break your spirit and one of its major tools in that war is humiliation. I read how former American soldier Bradley Manning (later Chelsea Manning after a sex-change) was treated after being jailed for supplying WikiLeaks with documents exposing the enormity of

government and elite mendacity. Manning was isolated in solitary confinement for eight months, put under 24-hour surveillance, forced to hand over clothing before going to bed, and stand naked for every roll call. This is systematic humiliation. The introduction of anal swab 'Covid' tests in China has been done for the same reason to delete self-respect and induce compliant submission. Anal swabs are mandatory for incoming passengers in parts of China and American diplomats have said they were forced to undergo the indignity which would have been calculated humiliation by the Cult-owned Chinese government that has America in its sights.

Government-people: An abusive relationship

Spirit-breaking psychological techniques include giving people hope and apparent respite from tyranny only to take it away again. This happened in the UK during Christmas, 2020, when the psycho-psychologists and their political lackeys announced an easing of restrictions over the holiday only to reimpose them almost immediately on the basis of yet another lie. There is a big psychological difference between getting used to oppression and being given hope of relief only to have that dashed. Psychologists know this and we have seen the technique used repeatedly. Then there is traumatising people before you introduce more extreme regulations that require compliance. A perfect case was the announcement by the dark and sinister Whitty and Vallance in the UK that 'new data' predicted that 4,000 could die every day over the winter of 2020/2021 if we did not lockdown again. I think they call it lying and after traumatising people with that claim out came Jackboot Johnson the next day with new curbs on human freedom. Psychologists know that a frightened and traumatised mind becomes suggestable to submission and behaviour reframing. Underpinning all this has been to make people fearful and suspicious of each other and see themselves as a potential danger to others. In league with deleted self-respect you have the perfect psychological recipe for self-loathing. The relationship between authority and public is now demonstrably the same as that of

subservience to an abusive partner. These are signs of an abusive relationship explained by psychologist Leslie Becker-Phelps:

Psychological and emotional abuse: Undermining a partner's self-worth with verbal attacks, name-calling, and belittling. Humiliating the partner in public, unjustly accusing them of having an affair, or interrogating them about their every behavior. Keeping partner confused or off balance by saying they were just kidding or blaming the partner for 'making' them act this way … Feigning in public that they care while turning against them in private. This leads to victims frequently feeling confused, incompetent, unworthy, hopeless, and chronically self-doubting. [Apply these techniques to how governments have treated the population since New Year, 2020, and the parallels are obvious.]

Physical abuse: The abuser might physically harm their partner in a range of ways, such as grabbing, hitting, punching, or shoving them. They might throw objects at them or harm them with a weapon. [Observe the physical harm imposed by masks, lockdown, and so on.]

Threats and intimidation: One way abusers keep their partners in line is by instilling fear. They might be verbally threatening, or give threatening looks or gestures. Abusers often make it known that they are tracking their partner's every move. They might destroy their partner's possessions, threaten to harm them, or threaten to harm their family members. Not surprisingly, victims of this abuse often feel anxiety, fear, and panic. [No words necessary.]

Isolation: Abusers often limit their partner's activities, forbidding them to talk or interact with friends or family. They might limit access to a car or even turn off their phone. All of this might be done by physically holding them against their will, but is often accomplished through psychological abuse and intimidation. The more isolated a person feels, the fewer resources they have to help gain perspective on their situation and to escape from it. [No words necessary.]

Economic abuse: Abusers often make their partners beholden to them for money by controlling access to funds of any kind. They might prevent their partner from getting a job or withhold access to money they earn from a job. This creates financial dependency that makes leaving the relationship very difficult. [See destruction of livelihoods and the proposed meagre 'guaranteed income' so long as you do whatever you are told.]

Using children: An abuser might disparage their partner's parenting skills, tell their children lies about their partner, threaten to take custody of their children, or threaten to harm their children. These tactics instil fear and often elicit compliance. [See reframed social service mafia and how children are being mercilessly abused by the state over 'Covid' while their parents look on too frightened to do anything.]

A further recurring trait in an abusive relationship is the abused blaming themselves for their abuse and making excuses for the abuser. We have the public blaming each other for lockdown abuse by government and many making excuses for the government while attacking those who challenge the government. How often we have heard authorities say that rules are being imposed or reimposed only because people have refused to 'behave' and follow the rules. We don't want to do it – it's *you*.

Renegade Minds are an antidote to all of these things. They will never concede their self-respect no matter what the circumstances. Even when apparent humiliation is heaped upon them they laugh in its face and reflect back the humiliation on the abuser where it belongs. Renegade Minds will never wear masks they know are only imposed to humiliate, suppress and damage both physically and psychologically. Consequences will take care of themselves and they will never break their spirit or cause them to concede to tyranny. UK newspaper columnist Peter Hitchens was one of the few in the mainstream media to speak out against lockdowns and forced vaccinations. He then announced he had taken the jab. He wanted to see family members abroad and he believed vaccine passports were inevitable even though they had not yet been introduced. Hitchens

has a questioning and critical mind, but not a Renegade one. If he had no amount of pressure would have made him concede. Hitchens excused his action by saying that the battle has been lost. Renegade Minds never accept defeat when freedom is at stake and even if they are the last one standing the self-respect of not submitting to tyranny is more important than any outcome or any consequence.

That's why Renegade Minds are the only minds that ever changed anything worth changing.

CHAPTER EIGHT

'Reframing' insanity

Insanity is relative. It depends on who has who locked in what cage
Ray Bradbury

'Reframing' a mind means simply to change its perception and behaviour. This can be done subconsciously to such an extent that subjects have no idea they have been 'reframed' while to any observer changes in behaviour and attitudes are obvious.

Human society is being reframed on a ginormous scale since the start of 2020 and here we have the reason why psychologists rather than doctors have been calling the shots. Ask most people who have succumbed to 'Covid' reframing if they have changed and most will say 'no'; but they *have* and fundamentally. The Cult's long-game has been preparing for these times since way back and crucial to that has been to prepare both population and officialdom mentally and emotionally. To use the mind-control parlance they had to reframe the population with a mentality that would submit to fascism and reframe those in government and law enforcement to impose fascism or at least go along with it. The result has been the fact-deleted mindlessness of 'Wokeness' and officialdom that has either enthusiastically or unquestioningly imposed global tyranny demanded by reframed politicians on behalf of psychopathic and deeply evil cultists. 'Cognitive reframing' identifies and challenges the way someone sees the world in the form of situations, experiences and emotions and then restructures those perceptions to view the same set of circumstances in a different way. This can have

benefits if the attitudes are personally destructive while on the other side it has the potential for individual and collective mind control which the subject has no idea has even happened.

Cognitive therapy was developed in the 1960s by Aaron T. Beck who was born in Rhode Island in 1921 as the son of Jewish immigrants from the Ukraine. He became interested in the techniques as a treatment for depression. Beck's daughter Judith S. Beck is prominent in the same field and they founded the Beck Institute for Cognitive Behavior Therapy in Philadelphia in 1994. Cognitive reframing, however, began to be used worldwide by those with a very dark agenda. The Cult reframes politicians to change their attitudes and actions until they are completely at odds with what they once appeared to stand for. The same has been happening to government administrators at all levels, law enforcement, military and the human population. Cultists love mind control for two main reasons: It allows them to control what people think, do and say to secure agenda advancement and, by definition, it calms their legendary insecurity and fear of the unexpected. I have studied mind control since the time I travelled America in 1996. I may have been talking to next to no one in terms of an audience in those years, but my goodness did I gather a phenomenal amount of information and knowledge about so many things including the techniques of mind control. I have described this in detail in other books going back to *The Biggest Secret* in 1998. I met a very large number of people recovering from MKUltra and its offshoots and successors and I began to see how these same techniques were being used on the population in general. This was never more obvious than since the 'Covid' hoax began.

Reframing the enforcers

I have observed over the last two decades and more the very clear transformation in the dynamic between the police, officialdom and the public. I tracked this in the books as the relationship mutated from one of serving the public to seeing them as almost the enemy and certainly a lower caste. There has always been a class divide

based on income and always been some psychopathic, corrupt, and big-I-am police officers. This was different. Wholesale change was unfolding in the collective dynamic; it was less about money and far more about position and perceived power. An us-and-them was emerging. Noses were lifted skyward by government administration and law enforcement and their attitude to the public they were *supposed* to be serving changed to one of increasing contempt, superiority and control. The transformation was so clear and widespread that it had to be planned. Collective attitudes and dynamics do not change naturally and organically that quickly on that scale. I then came across an organisation in Britain called Common Purpose created in the late 1980s by Julia Middleton who would work in the office of Deputy Prime Minister John Prescott during the long and disastrous premiership of war criminal Tony Blair. When Blair speaks the Cult is speaking and the man should have been in jail a long time ago. Common Purpose proclaims itself to be one of the biggest 'leadership development' organisations in the world while functioning as a *charity* with all the financial benefits which come from that. It hosts 'leadership development' courses and programmes all over the world and claims to have 'brought together' what it calls 'leaders' from more than 100 countries on six continents. The modus operandi of Common Purpose can be compared with the work of the UK government's reframing network that includes the Behavioural Insights Team 'nudge unit' and 'Covid' reframing specialists at SPI-B. WikiLeaks described Common Purpose long ago as 'a hidden virus in our government and schools' which is unknown to the general public: 'It recruits and trains "leaders" to be loyal to the directives of Common Purpose and the EU, instead of to their own departments, which they then undermine or subvert, the NHS [National Health Service] being an example.' This is a vital point to understand the 'Covid' hoax. The NHS, and its equivalent around the world, has been utterly reframed in terms of administrators and much of the medical personnel with the transformation underpinned by recruitment policies. The outcome has been the criminal and psychopathic behaviour of the

NHS over 'Covid' and we have seen the same in every other major country. WikiLeaks said Common Purpose trainees are 'learning to rule without regard to democracy' and to usher in a police state (current events explained). Common Purpose operated like a 'glue' and had members in the NHS, BBC, police, legal profession, church, many of Britain's 7,000 quangos, local councils, the Civil Service, government ministries and Parliament, and controlled many RDA's (Regional Development Agencies). Here we have one answer for how and why British institutions and their like in other countries have changed so negatively in relation to the public. This further explains how and why the beyond-disgraceful reframed BBC has become a propaganda arm of 'Covid' fascism. They are all part of a network pursuing the same goal.

By 2019 Common Purpose was quoting a figure of 85,000 'leaders' that had attended its programmes. These 'students' of all ages are known as Common Purpose 'graduates' and they consist of government, state and local government officials and administrators, police chiefs and officers, and a whole range of others operating within the national, local and global establishment. Cressida Dick, Commissioner of the London Metropolitan Police, is the Common Purpose graduate who was the 'Gold Commander' that oversaw what can only be described as the murder of Brazilian electrician Jean Charles de Menezes in 2005. He was held down by psychopathic police and shot seven times in the head by a psychopathic lunatic after being mistaken for a terrorist when he was just a bloke going about his day. Dick authorised officers to pursue and keep surveillance on de Menezes and ordered that he be stopped from entering the underground train system. Police psychopaths took her at her word clearly. She was 'disciplined' for this outrage by being *promoted* – eventually to the top of the 'Met' police where she has been a disaster. Many Chief Constables controlling the police in different parts of the UK are and have been Common Purpose graduates. I have heard the 'graduate' network described as a sort of Mafia or secret society operating within the fabric of government at all levels pursuing a collective policy

ingrained at Common Purpose training events. Founder Julia Middleton herself has said:

> Locally and internationally, Common Purpose graduates will be 'lighting small fires' to create change in their organisations and communities … The Common Purpose effect is best illustrated by the many stories of small changes brought about by leaders, who themselves have changed.

A Common Purpose mission statement declared:

> Common Purpose aims to improve the way society works by expanding the vision, decision-making ability and influence of all kinds of leaders. The organisation runs a variety of educational programmes for leaders of all ages, backgrounds and sectors, in order to provide them with the inspirational, information and opportunities they need to change the world.

Yes, but into what? Since 2020 the answer has become clear.

NLP and the Delphi technique

Common Purpose would seem to be a perfect name or would common programming be better? One of the foundation methods of reaching 'consensus' (group think) is by setting the agenda theme and then encouraging, cajoling or pressuring everyone to agree a 'consensus' in line with the core theme promoted by Common Purpose. The methodology involves the 'Delphi technique', or an adaption of it, in which opinions are expressed that are summarised by a 'facilitator or change agent' at each stage. Participants are 'encouraged' to modify their views in the light of what others have said. Stage by stage the former individual opinions are merged into group consensus which just happens to be what Common Purpose wants them to believe. A key part of this is to marginalise anyone refusing to concede to group think and turn the group against them to apply pressure to conform. We are seeing this very technique used on the general population to make 'Covid' group-thinkers hostile to those who have seen through the bullshit. People can be reframed by using perception manipulation methods such as Neuro-Linguistic Programming (NLP) in which you change perception with the use of

carefully constructed language. An NLP website described the technique this way:

… A method of influencing brain behaviour (the 'neuro' part of the phrase) through the use of language (the 'linguistic' part) and other types of communication to enable a person to 'recode' the way the brain responds to stimuli (that's the 'programming') and manifest new and better behaviours. Neuro-Linguistic Programming often incorporates hypnosis and self-hypnosis to help achieve the change (or 'programming') that is wanted.

British alternative media operation UKColumn has done very detailed research into Common Purpose over a long period. I quoted co-founder and former naval officer Brian Gerrish in my book *Remember Who You Are*, published in 2011, as saying the following years before current times:

It is interesting that many of the mothers who have had children taken by the State speak of the Social Services people being icily cool, emotionless and, as two ladies said in slightly different words, '… like little robots'. We know that NLP is cumulative, so people can be given small imperceptible doses of NLP in a course here, another in a few months, next year etc. In this way, major changes are accrued in their personality, but the day by day change is almost unnoticeable.

In these and other ways 'graduates' have had their perceptions uniformly reframed and they return to their roles in the institutions of government, law enforcement, legal profession, military, 'education', the UK National Health Service and the whole swathe of the establishment structure to pursue a common agenda preparing for the 'post-industrial', 'post-democratic' society. I say 'preparing' but we are now there. 'Post-industrial' is code for the Great Reset and 'post-democratic' is 'Covid' fascism. UKColumn has spoken to partners of those who have attended Common Purpose 'training'. They have described how personalities and attitudes of 'graduates' changed very noticeably for the worse by the time they had completed the course. They had been 'reframed' and told they are the 'leaders' – the special ones – who know better than the population. There has also been the very demonstrable recruitment of psychopaths and narcissists into government administration at all

levels and law enforcement. If you want psychopathy hire psychopaths and you get a simple cause and effect. If you want administrators, police officers and 'leaders' to perceive the public as lesser beings who don't matter then employ narcissists. These personalities are identified using 'psychometrics' that identifies knowledge, abilities, attitudes and personality traits, mostly through carefully-designed questionnaires and tests. As this policy has passed through the decades we have had power-crazy, power-trippers appointed into law enforcement, security and government administration in preparation for current times and the dynamic between public and law enforcement/officialdom has been transformed. UKColumn's Brian Gerrish said of the narcissistic personality:

> Their love of themselves and power automatically means that they will crush others who get in their way. I received a major piece of the puzzle when a friend pointed out that when they made public officials re-apply for their own jobs several years ago they were also required to do psychometric tests. This was undoubtedly the start of the screening process to get 'their' sort of people in post.

How obvious that has been since 2020 although it was clear what was happening long before if people paid attention to the changing public-establishment dynamic.

Change agents

At the centre of events in 'Covid' Britain is the National Health Service (NHS) which has behaved disgracefully in slavishly following the Cult agenda. The NHS management structure is awash with Common Purpose graduates or 'change agents' working to a common cause. Helen Bevan, a Chief of Service Transformation at the NHS Institute for Innovation and Improvement, co-authored a document called 'Towards a million change agents, a review of the social movements literature: implications for large scale change in the NHS'. The document compared a project management approach to that of change and social movements where 'people change

themselves and each other – peer to peer'. Two definitions given for a 'social movement' were:

A group of people who consciously attempt to build a radically new social order; involves people of a broad range of social backgrounds; and deploys politically confrontational and socially disruptive tactics – Cyrus Zirakzadeh 1997

Collective challenges, based on common purposes and social solidarities, in sustained interaction with elites, opponents, and authorities – Sidney Tarrow 1994

Helen Bevan wrote another NHS document in which she defined 'framing' as 'the process by which leaders construct, articulate and put across their message in a powerful and compelling way in order to win people to their cause and call them to action'. I think I could come up with another definition that would be rather more accurate. The National Health Service and institutions of Britain and the wider world have been taken over by reframed 'change agents' and that includes everything from the United Nations to national governments, local councils and social services which have been kidnapping children from loving parents on an extraordinary and gathering scale on the road to the end of parenthood altogether. Children from loving homes are stolen and kidnapped by the state and put into the 'care' (inversion) of the local authority through council homes, foster parents and forced adoption. At the same time children are allowed to be abused without response while many are under council 'care'. UKColumn highlighted the Common Purpose connection between South Yorkshire Police and Rotherham council officers in the case of the scandal in that area of the sexual exploitation of children to which the authorities turned not one blind eye, but both:

We were alarmed to discover that the Chief Executive, the Strategic Director of Children and Young People's Services, the Manager for the Local Strategic Partnership, the Community Cohesion Manager, the Cabinet Member for Cohesion, the Chief Constable and his predecessor had all attended Leadership training courses provided by the pseudo-charity Common Purpose.

Once 'change agents' have secured positions of hire and fire within any organisation things start to move very quickly. Personnel are then hired and fired on the basis of whether they will work towards the agenda the change agent represents. If they do they are rapidly promoted even though they may be incompetent. Those more qualified and skilled who are pre-Common Purpose 'old school' see their careers stall and even disappear. This has been happening for decades in every institution of state, police, 'health' and social services and all of them have been transformed as a result in their attitudes to their jobs and the public. Medical professions, including nursing, which were once vocations for the caring now employ many cold, callous and couldn't give a shit personality types. The UKColumn investigation concluded:

By blurring the boundaries between people, professions, public and private sectors, responsibility and accountability, Common Purpose encourages 'graduates' to believe that as new selected leaders, they can work together, outside of the established political and social structures, to achieve a paradigm shift or CHANGE – so called 'Leading Beyond Authority'. In doing so, the allegiance of the individual becomes 'reframed' on CP colleagues and their NETWORK.

Reframing the Face-Nappies

Nowhere has this process been more obvious than in the police where recruitment of psychopaths and development of unquestioning mind-controlled group-thinkers have transformed law enforcement into a politically-correct 'Woke' joke and a travesty of what should be public service. Today they wear their face-nappies like good little gofers and enforce 'Covid' rules which are fascism under another name. Alongside the specifically-recruited psychopaths we have software minds incapable of free thought. Brian Gerrish again:

An example is the policeman who would not get on a bike for a press photo because he had not done the cycling proficiency course. Normal people say this is political correctness gone mad. Nothing could be further from the truth. The policeman has been reframed, and in his reality it is perfect common sense not to get on the bike 'because he hasn't done the cycling course'.

Another example of this is where the police would not rescue a boy from a pond until they had taken advice from above on the 'risk assessment'. A normal person would have arrived, perhaps thought of the risk for a moment, and dived in. To the police now 'reframed', they followed 'normal' procedure.

There are shocking cases of reframed ambulance crews doing the same. Sheer unthinking stupidity of London Face-Nappies headed by Common Purpose graduate Cressida Dick can be seen in their behaviour at a vigil in March, 2021, for a murdered woman, Sarah Everard. A police officer had been charged with the crime. Anyone with a brain would have left the vigil alone in the circumstances. Instead they 'manhandled' women to stop them breaking 'Covid rules' to betray classic reframing. Minds in the thrall of perception control have no capacity for seeing a situation on its merits and acting accordingly. 'Rules is rules' is their only mind-set. My father used to say that rules and regulations are for the guidance of the intelligent and the blind obedience of the idiot. Most of the intelligent, decent, coppers have gone leaving only the other kind and a few old school for whom the job must be a daily nightmare. The combination of psychopaths and rule-book software minds has been clearly on public display in the 'Covid' era with automaton robots in uniform imposing fascistic 'Covid' regulations on the population without any personal initiative or judging situations on their merits. There are thousands of examples around the world, but I'll make my point with the infamous Derbyshire police in the English East Midlands – the ones who think pouring dye into beauty spots and using drones to track people walking in the countryside away from anyone is called 'policing'. To them there are rules decreed by the government which they have to enforce and in their bewildered state a group gathering in a closed space and someone walking alone in the countryside are the same thing. It is beyond idiocy and enters the realm of clinical insanity.

Police officers in Derbyshire said they were 'horrified' – *horrified* – to find 15 to 20 'irresponsible' kids playing a football match at a closed leisure centre 'in breach of coronavirus restrictions'. When they saw the police the kids ran away leaving their belongings behind and the reframed men and women of Derbyshire police were seeking to establish their identities with a view to fining their parents. The most natural thing for youngsters to do – kicking a ball about – is turned into a criminal activity and enforced by the moronic software programs of Derbyshire police. You find the same mentality in every country. These barely conscious 'horrified' officers said they had to take action because 'we need to ensure these rules are being followed' and 'it is of the utmost importance that you ensure your children are following the rules and regulations for Covid-19'. Had any of them done ten seconds of research to see if this parroting of their masters' script could be supported by any evidence? Nope. Reframed people don't think – others think for them and that's the whole idea of reframing. I have seen police officers one after the other repeating without question word for word what officialdom tells them just as I have seen great swathes of the public doing the same. Ask either for 'their' opinion and out spews what they have been told to think by the official narrative. Police and public may seem to be in different groups, but their mentality is the same. Most people do whatever they are told in fear not doing so or because they believe what officialdom tells them; almost the entirety of the police do what they are told for the same reason. Ultimately it's the tiny inner core of the global Cult that's telling both what to do.

So Derbyshire police were 'horrified'. Oh, really? Why did they think those kids were playing football? It was to relieve the psychological consequences of lockdown and being denied human contact with their friends and interaction, touch and discourse vital to human psychological health. Being denied this month after month has dismantled the psyche of many children and young people as depression and suicide have exploded. Were Derbyshire police *horrified by that*? Are you kidding? Reframed people don't have those

mental and emotional processes that can see how the impact on the psychological health of youngsters is far more dangerous than any 'virus' even if you take the mendacious official figures to be true. The reframed are told (programmed) how to act and so they do. The Derbyshire Chief Constable in the first period of lockdown when the black dye and drones nonsense was going on was Peter Goodman. He was the man who severed the connection between his force and the Derbyshire Constabulary *Male Voice* Choir when he decided that it was not inclusive enough to allow women to join. The fact it was a male voice choir making a particular sound produced by male voices seemed to elude a guy who terrifyingly ran policing in Derbyshire. He retired weeks after his force was condemned as disgraceful by former Supreme Court Justice Jonathan Sumption for their behaviour over extreme lockdown impositions. Goodman was replaced by his deputy Rachel Swann who was in charge when her officers were 'horrified'. The police statement over the boys committing the hanging-offence of playing football included the line about the youngsters being 'irresponsible in the times we are all living through' missing the point that the real relevance of the 'times we are all living through' is the imposition of fascism enforced by psychopaths and reframed minds of police officers playing such a vital part in establishing the fascist tyranny that their own children and grandchildren will have to live in their entire lives. As a definition of insanity that is hard to beat although it might be run close by imposing masks on people that can have a serious effect on their health while wearing a face nappy all day themselves. Once again public and police do it for the same reason – the authorities tell them to and who are they to have the self-respect to say no?

Wokers in uniform

How reframed do you have to be to arrest a *six-year-old* and take him to court for *picking a flower* while waiting for a bus? Brain dead police and officialdom did just that in North Carolina where criminal proceedings happen regularly for children under nine. Attorney Julie Boyer gave the six-year-old crayons and a colouring book

during the 'flower' hearing while the 'adults' decided his fate. County Chief District Court Judge Jay Corpening asked: 'Should a child that believes in Santa Claus, the Easter Bunny and the tooth fairy be making life-altering decisions?' Well, of course not, but common sense has no meaning when you have a common purpose and a reframed mind. Treating children in this way, and police operating in American schools, is all part of the psychological preparation for children to accept a police state as normal all their adult lives. The same goes for all the cameras and biometric tracking technology in schools. Police training is focused on reframing them as snowflake Wokers and this is happening in the military. Pentagon top brass said that 'training sessions on extremism' were needed for troops who asked why they were so focused on the Capitol Building riot when Black Lives Matter riots were ignored. What's the difference between them some apparently and rightly asked. Actually, there is a difference. Five people died in the Capitol riot, only one through violence, and that was a police officer shooting an unarmed protestor. BLM riots killed at least 25 people and cost billions. Asking the question prompted the psychopaths and reframed minds that run the Pentagon to say that more 'education' (programming) was needed. Troop training is all based on psychological programming to make them fodder for the Cult – 'Military men are just dumb, stupid animals to be used as pawns in foreign policy' as Cult-to-his-DNA former Secretary of State Henry Kissinger famously said. Governments see the police in similar terms and it's time for those among them who can see this to defend the people and stop being enforcers of the Cult agenda upon the people.

The US military, like the country itself, is being targeted for destruction through a long list of Woke impositions. Cult-owned gaga 'President' Biden signed an executive order when he took office to allow taxpayer money to pay for transgender surgery for active military personnel and veterans. Are you a man soldier? No, I'm a LGBTQIA+ with a hint of Skoliosexual and Spectrasexual. Oh, good man. Bad choice of words you bigot. The Pentagon announced in March, 2021, the appointment of the first 'diversity and inclusion

officer' for US Special Forces. Richard Torres-Estrada arrived with the publication of a 'D&I Strategic Plan which will guide the enterprise-wide effort to institutionalize and sustain D&I'. If you think a Special Forces 'Strategic Plan' should have something to do with defending America you haven't been paying attention. Defending Woke is now the military's new role. Torres-Estrada has posted images comparing Donald Trump with Adolf Hitler and we can expect no bias from him as a representative of the supposedly non-political Pentagon. Cable news host Tucker Carlson said: 'The Pentagon is now the Yale faculty lounge but with cruise missiles.' Meanwhile Secretary of Defense Lloyd Austin, a board member of weapons-maker Raytheon with stock and compensation interests in October, 2020, worth $1.4 million, said he was purging the military of the 'enemy within' – anyone who isn't Woke and supports Donald Trump. Austin refers to his targets as 'racist extremists' while in true Woke fashion being himself a racist extremist. Pentagon documents pledge to 'eradicate, eliminate and conquer all forms of racism, sexism and homophobia'. The definitions of these are decided by 'diversity and inclusion committees' peopled by those who see racism, sexism and homophobia in every situation and opinion. Woke (the Cult) is dismantling the US military and purging testosterone as China expands its military and gives its troops 'masculinity training'. How do we think that is going to end when this is all Cult coordinated? The US military, like the British military, is controlled by Woke and spineless top brass who just go along with it out of personal career interests.

'Woke' means fast asleep

Mind control and perception manipulation techniques used on individuals to create group-think have been unleashed on the global population in general. As a result many have no capacity to see the obvious fascist agenda being installed all around them or what 'Covid' is really all about. Their brains are firewalled like a computer system not to process certain concepts, thoughts and realisations that are bad for the Cult. The young are most targeted as the adults they

will be when the whole fascist global state is planned to be fully implemented. They need to be prepared for total compliance to eliminate all pushback from entire generations. The Cult has been pouring billions into taking complete control of 'education' from schools to universities via its operatives and corporations and not least Bill Gates as always. The plan has been to transform 'education' institutions into programming centres for the mentality of 'Woke'. James McConnell, professor of psychology at the University of Michigan, wrote in *Psychology Today* in 1970:

> The day has come when we can combine sensory deprivation with drugs, hypnosis, and astute manipulation of reward and punishment, to gain almost absolute control over an individual's behaviour. It should then be possible to achieve a very rapid and highly effective type of brainwashing that would allow us to make dramatic changes in a person's behaviour and personality ...
>
> … We should reshape society so that we all would be trained from birth to want to do what society wants us to do. We have the techniques to do it... no-one owns his own personality you acquired, and there's no reason to believe you should have the right to refuse to acquire a new personality if your old one is anti-social.

This was the potential for mass brainwashing in 1970 and the mentality there displayed captures the arrogant psychopathy that drives it forward. I emphasise that not all young people have succumbed to Woke programming and those that haven't are incredibly impressive people given that today's young are the most perceptually-targeted generations in history with all the technology now involved. Vast swathes of the young generations, however, have fallen into the spell – and that's what it is – of Woke. The Woke mentality and perceptual program is founded on *inversion* and you will appreciate later why that is so significant. Everything with Woke is inverted and the opposite of what it is claimed to be. Woke was a term used in African-American culture from the 1900s and referred to an awareness of social and racial justice. This is not the meaning of the modern version or 'New Woke' as I call it in *The Answer*. Oh, no, Woke today means something very different no matter how much Wokers may seek to hide that and insist Old Woke and New

Woke are the same. See if you find any 'awareness of social justice' here in the modern variety:

- Woke demands 'inclusivity' while excluding anyone with a different opinion and calls for mass censorship to silence other views.

- Woke claims to stand against oppression when imposing oppression is the foundation of all that it does. It is the driver of political correctness which is nothing more than a Cult invention to manipulate the population to silence itself.

- Woke believes itself to be 'liberal' while pursuing a global society that can only be described as fascist (see 'anti-fascist' fascist Antifa).

- Woke calls for 'social justice' while spreading injustice wherever it goes against the common 'enemy' which can be easily identified as a differing view.

- Woke is supposed to be a metaphor for 'awake' when it is solid-gold asleep and deep in a Cult-induced coma that meets the criteria for 'off with the fairies'.

I state these points as obvious facts if people only care to look. I don't do this with a sense of condemnation. We need to appreciate that the onslaught of perceptual programming on the young has been incessant and merciless. I can understand why so many have been reframed, or, given their youth, framed from the start to see the world as the Cult demands. The Cult has had access to their minds day after day in its 'education' system for their entire formative years. Perception is formed from information received and the Cult-created system is a life-long download of information delivered to elicit a particular perception, thus behaviour. The more this has expanded into still new extremes in recent decades and ever-increasing censorship has deleted other opinions and information why wouldn't that lead to a perceptual reframing on a mass scale? I

have described already cradle-to-grave programming and in more recent times the targeting of young minds from birth to adulthood has entered the stratosphere. This has taken the form of skewing what is 'taught' to fit the Cult agenda and the omnipresent techniques of group-think to isolate non-believers and pressure them into line. There has always been a tendency to follow the herd, but we really are in a new world now in relation to that. We have parents who can see the 'Covid' hoax told by their children not to stop them wearing masks at school, being 'Covid' tested or having the 'vaccine' in fear of the peer-pressure consequences of being different. What is 'peer-pressure' if not pressure to conform to group-think? Renegade Minds never group-think and always retain a set of perceptions that are unique to them. Group-think is always underpinned by consequences for not group-thinking. Abuse now aimed at those refusing DNA-manipulating 'Covid vaccines' are a potent example of this. The biggest pressure to conform comes from the very group which is itself being manipulated. 'I am programmed to be part of a hive mind and so you must be.'

Woke control structures in 'education' now apply to every mainstream organisation. Those at the top of the 'education' hierarchy (the Cult) decide the policy. This is imposed on governments through the Cult network; governments impose it on schools, colleges and universities; their leadership impose the policy on teachers and academics and they impose it on children and students. At any level where there is resistance, perhaps from a teacher or university lecturer, they are targeted by the authorities and often fired. Students themselves regularly demand the dismissal of academics (increasingly few) at odds with the narrative that the students have been programmed to believe in. It is quite a thought that students who are being targeted by the Cult become so consumed by programmed group-think that they launch protests and demand the removal of those who are trying to push back against those targeting the students. Such is the scale of perceptual inversion. We see this with 'Covid' programming as the Cult imposes the rules via psycho-psychologists and governments on

shops, transport companies and businesses which impose them on their staff who impose them on their customers who pressure Pushbackers to conform to the will of the Cult which is in the process of destroying them and their families. Scan all aspects of society and you will see the same sequence every time.

Fact free Woke and hijacking the 'left'

There is no more potent example of this than 'Woke', a mentality only made possible by the deletion of factual evidence by an 'education' system seeking to produce an ever more uniform society. Why would you bother with facts when you don't know any? Deletion of credible history both in volume and type is highly relevant. Orwell said: 'Who controls the past controls the future: who controls the present controls the past.' They who control the perception of the past control the perception of the future and they who control the present control the perception of the past through the writing and deleting of history. Why would you oppose the imposition of Marxism in the name of Wokeism when you don't know that Marxism cost at least 100 million lives in the 20th century alone? Watch videos and read reports in which Woker generations are asked basic historical questions – it's mind-blowing. A survey of 2,000 people found that six percent of millennials (born approximately early1980s to early 2000s) believed the Second World War (1939-1945) broke out with the assassination of President Kennedy (in 1963) and one in ten thought Margaret Thatcher was British Prime Minister at the time. She was in office between 1979 and 1990. We are in a post-fact society. Provable facts are no defence against the fascism of political correctness or Silicon Valley censorship. Facts don't matter anymore as we have witnessed with the 'Covid' hoax. Sacrificing uniqueness to the Woke group-think religion is all you are required to do and that means thinking for yourself is the biggest Woke no, no. All religions are an expression of group-think and censorship and Woke is just another religion with an orthodoxy defended by group-think and censorship. Burned at

the stake becomes burned on Twitter which leads back eventually to burned at the stake as Woke humanity regresses to ages past.

The biggest Woke inversion of all is its creators and funders. I grew up in a traditional left of centre political household on a council estate in Leicester in the 1950s and 60s – you know, the left that challenged the power of wealth-hoarding elites and threats to freedom of speech and opinion. In those days students went on marches defending freedom of speech while today's Wokers march for its deletion. What on earth could have happened? Those very elites (collectively the Cult) that we opposed in my youth and early life have funded into existence the antithesis of that former left and hijacked the 'brand' while inverting everything it ever stood for. We have a mentality that calls itself 'liberal' and 'progressive' while acting like fascists. Cult billionaires and their corporations have funded themselves into control of 'education' to ensure that Woke programming is unceasing throughout the formative years of children and young people and that non-Wokers are isolated (that word again) whether they be students, teachers or college professors. The Cult has funded into existence the now colossal global network of Woke organisations that have spawned and promoted all the 'causes' on the Cult wish-list for global transformation and turned Wokers into demanders of them. Does anyone really think it's a coincidence that the Cult agenda for humanity is a carbon (sorry) copy of the societal transformations desired by Woke?? These are only some of them:

Political correctness: The means by which the Cult deletes all public debates that it knows it cannot win if we had the free-flow of information and evidence.

Human-caused 'climate change': The means by which the Cult seeks to transform society into a globally-controlled dictatorship imposing its will over the fine detail of everyone's lives 'to save the planet' which doesn't actually need saving.

Transgender obsession: Preparing collective perception to accept the 'new human' which would not have genders because it would be created technologically and not through procreation. I'll have much more on this in Human 2.0.

Race obsession: The means by which the Cult seeks to divide and rule the population by triggering racial division through the perception that society is more racist than ever when the opposite is the case. Is it perfect in that regard? No. But to compare today with the racism of apartheid and segregation brought to an end by the civil rights movement in the 1960s is to insult the memory of that movement and inspirations like Martin Luther King. Why is the 'anti-racism' industry (which it is) so dominated by privileged white people?

White supremacy: This is a label used by privileged white people to demonise poor and deprived white people pushing back on tyranny to marginalise and destroy them. White people are being especially targeted as the dominant race by number within Western society which the Cult seeks to transform in its image. If you want to change a society you must weaken and undermine its biggest group and once you have done that by using the other groups you next turn on them to do the same … 'Then they came for the Jews and I was not a Jew so I did nothing.'

Mass migration: The mass movement of people from the Middle East, Africa and Asia into Europe, from the south into the United States and from Asia into Australia are another way the Cult seeks to dilute the racial, cultural and political influence of white people on Western society. White people ask why their governments appear to be working against them while being politically and culturally biased towards incoming cultures. Well, here's your answer. In the same way sexually 'straight' people, men and women, ask why the

authorities are biased against them in favour of other sexualities. The answer is the same – that's the way the Cult wants it to be for very sinister motives.

These are all central parts of the Cult agenda and central parts of the Woke agenda and Woke was created and continues to be funded to an immense degree by Cult billionaires and corporations. If anyone begins to say 'coincidence' the syllables should stick in their throat.

Billionaire 'social justice warriors'

Joe Biden is a 100 percent-owned asset of the Cult and the Wokers' man in the White House whenever he can remember his name and for however long he lasts with his rapidly diminishing cognitive function. Even walking up the steps of an aircraft without falling on his arse would appear to be a challenge. He's not an empty-shell puppet or anything. From the minute Biden took office (or the Cult did) he began his executive orders promoting the Woke wish-list. You will see the Woke agenda imposed ever more severely because it's really the *Cult* agenda. Woke organisations and activist networks spawned by the Cult are funded to the extreme so long as they promote what the Cult wants to happen. Woke is funded to promote 'social justice' by billionaires who become billionaires by destroying social justice. The social justice mantra is only a cover for dismantling social justice and funded by billionaires that couldn't give a damn about social justice. Everything makes sense when you see that. One of Woke's premier funders is Cult billionaire financier George Soros who said: 'I am basically there to make money, I cannot and do not look at the social consequences of what I do.' This is the same Soros who has given more than $32 billion to his Open Society Foundations global Woke network and funded Black Lives Matter, mass immigration into Europe and the United States, transgender activism, climate change activism, political correctness and groups targeting 'white supremacy' in the form of privileged white thugs that dominate Antifa. What a scam it all is and when

you are dealing with the unquestioning fact-free zone of Woke scamming them is child's play. All you need to pull it off in all these organisations are a few in-the-know agents of the Cult and an army of naïve, reframed, uninformed, narcissistic, know-nothings convinced of their own self-righteousness, self-purity and virtue.

Soros and fellow billionaires and billionaire corporations have poured hundreds of millions into Black Lives Matter and connected groups and promoted them to a global audience. None of this is motivated by caring about black people. These are the billionaires that have controlled and exploited a system that leaves millions of black people in abject poverty and deprivation which they do absolutely nothing to address. The same Cult networks funding BLM were behind the *slave trade!* Black Lives Matter hijacked a phrase that few would challenge and they have turned this laudable concept into a political weapon to divide society. You know that BLM is a fraud when it claims that *All* Lives Matter, the most inclusive statement of all, is 'racist'. BLM and its Cult masters don't want to end racism. To them it's a means to an end to control all of humanity never mind the colour, creed, culture or background. What has destroying the nuclear family got to do with ending racism? Nothing – but that is one of the goals of BLM and also happens to be a goal of the Cult as I have been exposing in my books for decades. Stealing children from loving parents and giving schools ever more power to override parents is part of that same agenda. BLM is a Marxist organisation and why would that not be the case when the Cult created Marxism *and* BLM? Patrisse Cullors, a BLM co-founder, said in a 2015 video that she and her fellow organisers, including co-founder Alicia Garza, are 'trained Marxists'. The lady known after marriage as Patrisse Khan-Cullors bought a $1.4 million home in 2021 in one of the whitest areas of California with a black population of just 1.6 per cent and has so far bought *four* high-end homes for a total of $3.2 million. How very Marxist. There must be a bit of spare in the BLM coffers, however, when Cult corporations and billionaires have handed over the best part of $100 million. Many black people can see that Black Lives Matter is not

working for them, but against them, and this is still more confirmation. Black journalist Jason Whitlock, who had his account suspended by Twitter for simply linking to the story about the 'Marxist's' home buying spree, said that BLM leaders are 'making millions of dollars off the backs of these dead black men who they wouldn't spit on if they were on fire and alive'.

Black Lies Matter

Cult assets and agencies came together to promote BLM in the wake of the death of career criminal George Floyd who had been jailed a number of times including for forcing his way into the home of a black woman with others in a raid in which a gun was pointed at her stomach. Floyd was filmed being held in a Minneapolis street in 2020 with the knee of a police officer on his neck and he subsequently died. It was an appalling thing for the officer to do, but the same technique has been used by police on peaceful protestors of lockdown without any outcry from the Woke brigade. As unquestioning supporters of the Cult agenda Wokers have supported lockdown and all the 'Covid' claptrap while attacking anyone standing up to the tyranny imposed in its name. Court documents would later include details of an autopsy on Floyd by County Medical Examiner Dr Andrew Baker who concluded that Floyd had taken a fatal level of the drug fentanyl. None of this mattered to fact-free, question-free, Woke. Floyd's death was followed by worldwide protests against police brutality amid calls to defund the police. Throwing babies out with the bathwater is a Woke speciality. In the wake of the murder of British woman Sarah Everard a Green Party member of the House of Lords, Baroness Jones of Moulescoomb (Nincompoopia would have been better), called for a 6pm curfew for all men. This would be in breach of the Geneva Conventions on war crimes which ban collective punishment, but that would never have crossed the black and white Woke mind of Baroness Nincompoopia who would have been far too convinced of her own self-righteousness to compute such details. Many American cities did defund the police in the face of Floyd riots

and after $15 million was deleted from the police budget in Washington DC under useless Woke mayor Muriel Bowser car-jacking alone rose by 300 percent and within six months the US capital recorded its highest murder rate in 15 years. The same happened in Chicago and other cities in line with the Cult/Soros plan to bring fear to streets and neighbourhoods by reducing the police, releasing violent criminals and not prosecuting crime. This is the mob-rule agenda that I have warned in the books was coming for so long. Shootings in the area of Minneapolis where Floyd was arrested increased by 2,500 percent compared with the year before. Defunding the police over George Floyd has led to a big increase in dead people with many of them black. Police protection for politicians making these decisions stayed the same or increased as you would expect from professional hypocrites. The Cult doesn't actually want to abolish the police. It wants to abolish local control over the police and hand it to federal government as the psychopaths advance the Hunger Games Society. Many George Floyd protests turned into violent riots with black stores and businesses destroyed by fire and looting across America fuelled by Black Lives Matter. Woke doesn't do irony. If you want civil rights you must loot the liquor store and the supermarket and make off with a smart TV. It's the only way.

It's not a race war – it's a class war

Black people are patronised by privileged blacks and whites alike and told they are victims of white supremacy. I find it extraordinary to watch privileged blacks supporting the very system and bloodline networks behind the slave trade and parroting the same Cult-serving manipulative crap of their privileged white, often billionaire, associates. It is indeed not a race war but a class war and colour is just a diversion. Black Senator Cory Booker and black Congresswoman Maxine Waters, more residents of Nincompoopia, personify this. Once you tell people they are victims of someone else you devalue both their own responsibility for their plight and the power they have to impact on their reality and experience. Instead

we have: 'You are only in your situation because of whitey – turn on them and everything will change.' It won't change. Nothing changes in our lives unless *we* change it. Crucial to that is never seeing yourself as a victim and always as the creator of your reality. Life is a simple sequence of choice and consequence. Make different choices and you create different consequences. *You* have to make those choices – not Black Lives Matter, the Woke Mafia and anyone else that seeks to dictate your life. Who are they these Wokers, an emotional and psychological road traffic accident, to tell you what to do? Personal empowerment is the last thing the Cult and its Black Lives Matter want black people or anyone else to have. They claim to be defending the underdog while *creating* and perpetuating the underdog. The Cult's worst nightmare is human unity and if they are going to keep blacks, whites and every other race under economic servitude and control then the focus must be diverted from what they have in common to what they can be manipulated to believe divides them. Blacks have to be told that their poverty and plight is the fault of the white bloke living on the street in the same poverty and with the same plight they are experiencing. The difference is that your plight black people is due to him, a white supremacist with 'white privilege' living on the street. Don't unite as one human family against your mutual oppressors and suppressors – fight the oppressor with the white face who is as financially deprived as you are. The Cult knows that as its 'Covid' agenda moves into still new levels of extremism people are going to respond and it has been spreading the seeds of disunity everywhere to stop a united response to the evil that targets *all of us*.

Racist attacks on 'whiteness' are getting ever more outrageous and especially through the American Democratic Party which has an appalling history for anti-black racism. Barack Obama, Joe Biden, Hillary Clinton and Nancy Pelosi all eulogised about Senator Robert Byrd at his funeral in 2010 after a nearly 60-year career in Congress. Byrd was a brutal Ku Klux Klan racist and a violent abuser of Cathy O'Brien in MKUltra. He said he would never fight in the military 'with a negro by my side' and 'rather I should die a thousand times,

and see Old Glory trampled in the dirt never to rise again, than to see this beloved land of ours become degraded by race mongrels, a throwback to the blackest specimen from the wilds'. Biden called Byrd a 'very close friend and mentor'. These 'Woke' hypocrites are not anti-racist they are anti-poor and anti-people not of their perceived class. Here is an illustration of the scale of anti-white racism to which we have now descended. Seriously Woke and moronic *New York Times* contributor Damon Young described whiteness as a 'virus' that 'like other viruses will not die until there are no bodies left for it to infect'. He went on: '… the only way to stop it is to locate it, isolate it, extract it, and kill it.' Young can say that as a black man with no consequences when a white man saying the same in reverse would be facing a jail sentence. *That's* racism. We had super-Woke numbskull senators Tammy Duckworth and Mazie Hirono saying they would object to future Biden Cabinet appointments if he did not nominate more Asian Americans and Pacific Islanders. Never mind the ability of the candidate what do they look like? Duckworth said: 'I will vote for racial minorities and I will vote for LGBTQ, but anyone else I'm not voting for.' Appointing people on the grounds of race is illegal, but that was not a problem for this ludicrous pair. They were on-message and that's a free pass in any situation.

Critical race racism

White children are told at school they are intrinsically racist as they are taught the divisive 'critical race theory'. This claims that the law and legal institutions are inherently racist and that race is a socially constructed concept used by white people to further their economic and political interests at the expense of people of colour. White is a 'virus' as we've seen. Racial inequality results from 'social, economic, and legal differences that white people create between races to maintain white interests which leads to poverty and criminality in minority communities'. I must tell that to the white guy sleeping on the street. The principal of East Side Community School in New York sent white parents a manifesto that called on

them to become 'white traitors' and advocate for full 'white abolition'. These people are teaching your kids when they urgently need a psychiatrist. The 'school' included a chart with 'eight white identities' that ranged from 'white supremacist' to 'white abolition' and defined the behaviour white people must follow to end 'the regime of whiteness'. Woke blacks and their privileged white associates are acting exactly like the slave owners of old and Ku Klux Klan racists like Robert Byrd. They are too full of their own self-purity to see that, but it's true. Racism is not a body type; it's a state of mind that can manifest through any colour, creed or culture.

Another racial fraud is *'equity'*. Not equality of treatment and opportunity – equity. It's a term spun as equality when it means something very different. Equality in its true sense is a raising up while 'equity' is a race to the bottom. Everyone in the same level of poverty is 'equity'. Keep everyone down – that's equity. The Cult doesn't want anyone in the human family to be empowered and BLM leaders, like all these 'anti-racist' organisations, continue their privileged, pampered existence by perpetuating the perception of gathering racism. When is the last time you heard an 'anti-racist' or 'anti-Semitism' organisation say that acts of racism and discrimination have *fallen?* It's not in the interests of their fund-raising and power to influence and the same goes for the professional soccer anti-racism operation, Kick It Out. Two things confirmed that the Black Lives Matter riots in the summer of 2020 were Cult creations. One was that while anti-lockdown protests were condemned in this same period for 'transmitting 'Covid' the authorities supported mass gatherings of Black Lives Matter supporters. I even saw self-deluding people claiming to be doctors say the two types of protest were not the same. No – the non-existent 'Covid' was in favour of lockdowns and attacked those that protested against them while 'Covid' supported Black Lives Matter and kept well away from its protests. The whole thing was a joke and as lockdown protestors were arrested, often brutally, by reframed Face-Nappies we had the grotesque sight of police officers taking the knee to Black Lives Matter, a Cult-funded Marxist

organisation that supports violent riots and wants to destroy the nuclear family and white people.

He's not white? Shucks!

Woke obsession with race was on display again when ten people were shot dead in Boulder, Colorado, in March, 2021. Cult-owned Woke TV channels like CNN said the shooter appeared to be a white man and Wokers were on Twitter condemning 'violent white men' with the usual mantras. Then the shooter's name was released as Ahmad Al Aliwi Alissa, an anti-Trump Arab-American, and the sigh of disappointment could be heard five miles away. Never mind that ten people were dead and what that meant for their families. Race baiting was all that mattered to these sick Cult-serving people like Barack Obama who exploited the deaths to further divide America on racial grounds which is his job for the Cult. This is the man that 'racist' white Americans made the first black president of the United States and then gave him a second term. Not-very-bright Obama has become filthy rich on the back of that and today appears to have a big influence on the Biden administration. Even so he's still a downtrodden black man and a victim of white supremacy. This disingenuous fraud reveals the contempt he has for black people when he puts on a Deep South Alabama accent whenever he talks to them, no, *at* them.

Another BLM red flag was how the now fully-Woke (fully-Cult) and fully-virtue-signalled professional soccer authorities had their teams taking the knee before every match in support of Marxist Black Lives Matter. Soccer authorities and clubs displayed 'Black Lives Matter' on the players' shirts and flashed the name on electronic billboards around the pitch. Any fans that condemned what is a Freemasonic taking-the-knee ritual were widely condemned as you would expect from the Woke virtue-signallers of professional sport and the now fully-Woke media. We have reverse racism in which you are banned from criticising any race or culture except for white people for whom anything goes – say what you like, no problem. What has this got to do with racial harmony and

equality? We've had black supremacists from Black Lives Matter telling white people to fall to their knees in the street and apologise for their white supremacy. Black supremacists acting like white supremacist slave owners of the past couldn't breach their self-obsessed, race-obsessed sense of self-purity. Joe Biden appointed a race-obsessed black supremacist Kristen Clarke to head the Justice Department Civil Rights Division. Clarke claimed that blacks are endowed with 'greater mental, physical and spiritual abilities' than whites. If anyone reversed that statement they would be vilified. Clarke is on-message so no problem. She's never seen a black-white situation in which the black figure is anything but a virtuous victim and she heads the Civil Rights Division which should treat everyone the same or it isn't civil rights. Another perception of the Renegade Mind: If something or someone is part of the Cult agenda they will be supported by Woke governments and media no matter what. If they're not, they will be condemned and censored. It really is that simple and so racist Clarke prospers despite (make that because of) her racism.

The end of culture

Biden's administration is full of such racial, cultural and economic bias as the Cult requires the human family to be divided into warring factions. We are now seeing racially-segregated graduations and everything, but everything, is defined through the lens of perceived 'racism. We have 'racist' mathematics, 'racist' food and even 'racist' *plants*. World famous Kew Gardens in London said it was changing labels on plants and flowers to tell its pre-'Covid' more than two million visitors a year how racist they are. Kew director Richard Deverell said this was part of an effort to 'move quickly to decolonise collections' after they were approached by one Ajay Chhabra 'an actor with an insight into how sugar cane was linked to slavery'. They are *plants* you idiots. 'Decolonisation' in the Woke manual really means colonisation of society with its mentality and by extension colonisation by the Cult. We are witnessing a new Chinese-style 'Cultural Revolution' so essential to the success of all

Marxist takeovers. Our cultural past and traditions have to be swept away to allow a new culture to be built-back-better. Woke targeting of long-standing Western cultural pillars including historical monuments and cancelling of historical figures is what happened in the Mao revolution in China which 'purged remnants of capitalist and traditional elements from Chinese society' and installed Maoism as the dominant ideology'. For China see the Western world today and for 'dominant ideology' see Woke. Better still see Marxism or Maoism. The 'Covid' hoax has specifically sought to destroy the arts and all elements of Western culture from people meeting in a pub or restaurant to closing theatres, music venues, sports stadiums, places of worship and even banning *singing*. Destruction of Western society is also why criticism of any religion is banned except for Christianity which again is the dominant religion as white is the numerically-dominant race. Christianity may be fading rapidly, but its history and traditions are weaved through the fabric of Western society. Delete the pillars and other structures will follow until the whole thing collapses. I am not a Christian defending that religion when I say that. I have no religion. It's just a fact. To this end Christianity has itself been turned Woke to usher its own downfall and its ranks are awash with 'change agents' – knowing and unknowing – at every level including Pope Francis (*definitely* knowing) and the clueless Archbishop of Canterbury Justin Welby (possibly not, but who can be sure?). Woke seeks to coordinate attacks on Western culture, traditions, and ways of life through 'intersectionality' defined as 'the complex, cumulative way in which the effects of multiple forms of discrimination (such as racism, sexism, and classism) combine, overlap, or intersect especially in the experiences of marginalised individuals or groups'. Wade through the Orwellian Woke-speak and this means coordinating disparate groups in a common cause to overthrow freedom and liberal values.

The entire structure of public institutions has been infested with Woke – government at all levels, political parties, police, military, schools, universities, advertising, media and trade unions. This abomination has been achieved through the Cult web by appointing

Wokers to positions of power and battering non-Wokers into line through intimidation, isolation and threats to their job. Many have been fired in the wake of the empathy-deleted, vicious hostility of 'social justice' Wokers and the desire of gutless, spineless employers to virtue-signal their Wokeness. Corporations are filled with Wokers today, most notably those in Silicon Valley. Ironically at the top they are not Woke at all. They are only exploiting the mentality their Cult masters have created and funded to censor and enslave while the Wokers cheer them on until it's their turn. Thus the Woke 'liberal left' is an inversion of the traditional liberal left. Campaigning for justice on the grounds of power and wealth distribution has been replaced by campaigning for identity politics. The genuine traditional left would never have taken money from today's billionaire abusers of fairness and justice and nor would the billionaires have wanted to fund that genuine left. It would not have been in their interests to do so. The division of opinion in those days was between the haves and have nots. This all changed with Cult manipulated and funded identity politics. The division of opinion today is between Wokers and non-Wokers and not income brackets. Cult corporations and their billionaires may have taken wealth disparity to cataclysmic levels of injustice, but as long as they speak the language of Woke, hand out the dosh to the Woke network and censor the enemy they are 'one of us'. Billionaires who don't give a damn about injustice are laughing at them till their bellies hurt. Wokers are not even close to self-aware enough to see that. The transformed 'left' dynamic means that Wokers who drone on about 'social justice' are funded by billionaires that have destroyed social justice the world over. It's *why* they are billionaires.

The climate con

Nothing encapsulates what I have said more comprehensively than the hoax of human-caused global warming. I have detailed in my books over the years how Cult operatives and organisations were the pump-primers from the start of the climate con. A purpose-built vehicle for this is the Club of Rome established by the Cult in 1968

with the Rockefellers and Rothschilds centrally involved all along. Their gofer frontman Maurice Strong, a Canadian oil millionaire, hosted the Earth Summit in Rio de Janeiro, Brazil, in 1992 where the global 'green movement' really expanded in earnest under the guiding hand of the Cult. The Earth Summit established Agenda 21 through the Cult-created-and-owned United Nations to use the illusion of human-caused climate change to justify the transformation of global society to save the world from climate disaster. It is a No-Problem-Reaction-Solution sold through governments, media, schools and universities as whole generations have been terrified into believing that the world was going to end in their lifetimes unless what old people had inflicted upon them was stopped by a complete restructuring of how everything is done. Chill, kids, it's all a hoax. Such restructuring is precisely what the Cult agenda demands (purely by coincidence of course). Today this has been given the codename of the Great Reset which is only an updated term for Agenda 21 and its associated Agenda 2030. The latter, too, is administered through the UN and was voted into being by the General Assembly in 2015. Both 21 and 2030 seek centralised control of all resources and food right down to the raindrops falling on your own land. These are some of the demands of Agenda 21 established in 1992. See if you recognise this society emerging today:

- End national sovereignty
- State planning and management of all land resources, ecosystems, deserts, forests, mountains, oceans and fresh water; agriculture; rural development; biotechnology; and ensuring *'equity'*
- The state to 'define the role' of business and financial resources
- Abolition of private property
- 'Restructuring' the family unit (see BLM)
- Children raised by the state
- People told what their job will be
- Major restrictions on movement
- Creation of 'human settlement zones'

- Mass resettlement as people are forced to vacate land where they live
- Dumbing down education
- Mass global depopulation in pursuit of all the above

The United Nations was created as a Trojan horse for world government. With the climate con of critical importance to promoting that outcome you would expect the UN to be involved. Oh, it's involved all right. The UN is promoting Agenda 21 and Agenda 2030 justified by 'climate change' while also driving the climate hoax through its Intergovernmental Panel on Climate Change (IPCC), one of the world's most corrupt organisations. The IPCC has been lying ferociously and constantly since the day it opened its doors with the global media hanging unquestioningly on its every mendacious word. The Green movement is entirely Woke and has long lost its original environmental focus since it was co-opted by the Cult. An obsession with 'global warming' has deleted its values and scrambled its head. I experienced a small example of what I mean on a beautiful country walk that I have enjoyed several times a week for many years. The path merged into the fields and forests and you felt at one with the natural world. Then a 'Green' organisation, the Hampshire and Isle of Wight Wildlife Trust, took over part of the land and proceeded to cut down a large number of trees, including mature ones, to install a horrible big, bright steel 'this-is-ours-stay-out' fence that destroyed the whole atmosphere of this beautiful place. No one with a feel for nature would do that. Day after day I walked to the sound of chainsaws and a magnificent mature weeping willow tree that I so admired was cut down at the base of the trunk. When I challenged a Woke young girl in a green shirt (of course) about this vandalism she replied: 'It's a weeping willow – it will grow back.' This is what people are paying for when they donate to the Hampshire and Isle of Wight Wildlife Trust and many other 'green' organisations today. It is not the environmental movement that I knew and instead has become a support-system – as with Extinction Rebellion – for a very dark agenda.

Private jets for climate justice

The Cult-owned, Gates-funded, World Economic Forum and its founder Klaus Schwab were behind the emergence of Greta Thunberg to harness the young behind the climate agenda and she was invited to speak to the world at … the UN. Schwab published a book, *Covid-19: The Great Reset* in 2020 in which he used the 'Covid' hoax and the climate hoax to lay out a new society straight out of Agenda 21 and Agenda 2030. Bill Gates followed in early 2021 when he took time out from destroying the world to produce a book in his name about the way to save it. Gates flies across the world in private jets and admitted that 'I probably have one of the highest greenhouse gas footprints of anyone on the planet … my personal flying alone is gigantic.' He has also bid for the planet's biggest private jet operator. Other climate change saviours who fly in private jets include John Kerry, the US Special Presidential Envoy for Climate, and actor Leonardo DiCaprio, a 'UN Messenger of Peace with special focus on climate change'. These people are so full of bullshit they could corner the market in manure. We mustn't be sceptical, though, because the Gates book, *How to Avoid a Climate Disaster: The Solutions We Have and the Breakthroughs We Need*, is a genuine attempt to protect the world and not an obvious pile of excrement attributed to a mega-psychopath aimed at selling his masters' plans for humanity. The Gates book and the other shite-pile by Klaus Schwab could have been written by the same person and may well have been. Both use 'climate change' and 'Covid' as the excuses for their new society and by coincidence the Cult's World Economic Forum and Bill and Melinda Gates Foundation promote the climate hoax and hosted Event 201 which pre-empted with a 'simulation' the very 'coronavirus' hoax that would be simulated for real on humanity within weeks. The British 'royal' family is promoting the 'Reset' as you would expect through Prince 'climate change caused the war in Syria' Charles and his hapless son Prince William who said that we must 'reset our relationship with nature and our trajectory as a species' to avoid a climate disaster. Amazing how many promotors of the 'Covid' and 'climate change' control

systems are connected to Gates and the World Economic Forum. A 'study' in early 2021 claimed that carbon dioxide emissions must fall by the equivalent of a global lockdown roughly every two years for the next decade to save the planet. The 'study' appeared in the same period that the Schwab mob claimed in a video that lockdowns destroying the lives of billions are good because they make the earth 'quieter' with less 'ambient noise'. They took down the video amid a public backlash for such arrogant, empathy-deleted stupidity You see, however, where they are going with this. Corinne Le Quéré, a professor at the Tyndall Centre for Climate Change Research, University of East Anglia, was lead author of the climate lockdown study, and she writes for … the World Economic Forum. Gates calls in 'his' book for changing 'every aspect of the economy' (long-time Cult agenda) and for humans to eat synthetic 'meat' (predicted in my books) while cows and other farm animals are eliminated. Australian TV host and commentator Alan Jones described what carbon emission targets would mean for farm animals in Australia alone if emissions were reduced as demanded by 35 percent by 2030 and zero by 2050:

Well, let's take agriculture, the total emissions from agriculture are about 75 million tonnes of carbon dioxide, equivalent. Now reduce that by 35 percent and you have to come down to 50 million tonnes, I've done the maths. So if you take for example 1.5 million cows, you're going to have to reduce the herd by 525,000 [by] 2030, nine years, that's 58,000 cows a year. The beef herd's 30 million, reduce that by 35 percent, that's 10.5 million, which means 1.2 million cattle have to go every year between now and 2030. This is insanity!

There are 75 million sheep. Reduce that by 35 percent, that's 26 million sheep, that's almost 3 million a year. So under the Paris Agreement over 30 million beasts. dairy cows, cattle, pigs and sheep would go. More than 8,000 every minute of every hour for the next decade, do these people know what they're talking about?

Clearly they don't at the level of campaigners, politicians and administrators. The Cult *does* know; that's the outcome it wants. We are faced with not just a war on humanity. Animals and the natural world are being targeted and I have been saying since the 'Covid' hoax began that the plan eventually was to claim that the 'deadly virus' is able to jump from animals, including farm animals and

domestic pets, to humans. Just before this book went into production came this story: 'Russia registers world's first Covid-19 vaccine for cats & dogs as makers of Sputnik V warn pets & farm animals could spread virus'. The report said 'top scientists warned that the deadly pathogen could soon begin spreading through homes and farms' and 'the next stage is the infection of farm and domestic animals'. Know the outcome and you'll see the journey. Think what that would mean for animals and keep your eye on a term called zoonosis or zoonotic diseases which transmit between animals and humans. The Cult wants to break the connection between animals and people as it does between people and people. Farm animals fit with the Cult agenda to transform food from natural to synthetic.

The gas of life is killing us

There can be few greater examples of Cult inversion than the condemnation of carbon dioxide as a dangerous pollutant when it is the gas of life. Without it the natural world would be dead and so we would all be dead. We breathe in oxygen and breathe out carbon dioxide while plants produce oxygen and absorb carbon dioxide. It is a perfect symbiotic relationship that the Cult wants to dismantle for reasons I will come to in the final two chapters. Gates, Schwab, other Cult operatives and mindless repeaters, want the world to be 'carbon neutral' by at least 2050 and the earlier the better. 'Zero carbon' is the cry echoed by lunatics calling for 'Zero Covid' when we already have it. These carbon emission targets will deindustrialise the world in accordance with Cult plans – the post-industrial, post-democratic society – and with so-called renewables like solar and wind not coming even close to meeting human energy needs blackouts and cold are inevitable. Texans got the picture in the winter of 2021 when a snow storm stopped wind turbines and solar panels from working and the lights went down along with water which relies on electricity for its supply system. Gates wants everything to be powered by electricity to ensure that his masters have the kill switch to stop all human activity, movement, cooking, water and warmth any time they like. The climate lie is so

stupendously inverted that it claims we must urgently reduce carbon dioxide when we *don't have enough*.

Co2 in the atmosphere is a little above 400 parts per million when the optimum for plant growth is 2,000 ppm and when it falls anywhere near 150 ppm the natural world starts to die and so do we. It fell to as low as 280 ppm in an 1880 measurement in Hawaii and rose to 413 ppm in 2019 with industrialisation which is why the planet has become *greener* in the industrial period. How insane then that psychopathic madman Gates is not satisfied only with blocking the rise of Co2. He's funding technology to suck it out of the atmosphere. The reason why will become clear. The industrial era is not destroying the world through Co2 and has instead turned around a potentially disastrous ongoing fall in Co2. Greenpeace co-founder and scientist Patrick Moore walked away from Greenpeace in 1986 and has exposed the green movement for fear-mongering and lies. He said that 500 million years ago there was *17 times* more Co2 in the atmosphere than we have today and levels have been falling for hundreds of millions of years. In the last 150 million years Co2 levels in Earth's atmosphere had reduced by *90 percent*. Moore said that by the time humanity began to unlock carbon dioxide from fossil fuels we were at '38 seconds to midnight' and in that sense: 'Humans are [the Earth's] salvation.' Moore made the point that only half the Co2 emitted by fossil fuels stays in the atmosphere and we should remember that all pollution pouring from chimneys that we are told is carbon dioxide is in fact nothing of the kind. It's pollution. Carbon dioxide is an invisible gas.

William Happer, Professor of Physics at Princeton University and long-time government adviser on climate, has emphasised the Co2 deficiency for maximum growth and food production. Greenhouse growers don't add carbon dioxide for a bit of fun. He said that most of the warming in the last 100 years, after the earth emerged from the super-cold period of the 'Little Ice Age' into a natural warming cycle, was over by 1940. Happer said that a peak year for warming in 1988 can be explained by a 'monster El Nino' which is a natural and cyclical warming of the Pacific that has nothing to do with 'climate

change'. He said the effect of Co2 could be compared to painting a wall with red paint in that once two or three coats have been applied it didn't matter how much more you slapped on because the wall will not get much redder. Almost all the effect of the rise in Co2 has already happened, he said, and the volume in the atmosphere would now have to *double* to increase temperature by a single degree. Climate hoaxers know this and they have invented the most ridiculously complicated series of 'feedback' loops to try to overcome this rather devastating fact. You hear puppet Greta going on cluelessly about feedback loops and this is why.

The Sun affects temperature? No you *climate denier*

Some other nonsense to contemplate: Climate graphs show that rises in temperature do not follow rises in Co2 – *it's the other way round* with a lag between the two of some 800 years. If we go back 800 years from present time we hit the Medieval Warm Period when temperatures were higher than now without any industrialisation and this was followed by the Little Ice Age when temperatures plummeted. The world was still emerging from these centuries of serious cold when many climate records began which makes the ever-repeated line of the 'hottest year since records began' meaningless when you are not comparing like with like. The coldest period of the Little Ice Age corresponded with the lowest period of sunspot activity when the Sun was at its least active. Proper scientists will not be at all surprised by this when it confirms the obvious fact that earth temperature is affected by the scale of Sun activity and the energetic power that it subsequently emits; but when is the last time you heard a climate hoaxer talking about the Sun as a source of earth temperature?? Everything has to be focussed on Co2 which makes up just 0.117 percent of so-called greenhouse gases and only a fraction of even that is generated by human activity. The rest is natural. More than *90 percent* of those greenhouse gases are water vapour and clouds (Fig 9). Ban moisture I say. Have you noticed that the climate hoaxers no longer use the polar bear as their promotion image? That's because far from becoming extinct polar

bear communities are stable or thriving. Joe Bastardi, American meteorologist, weather forecaster and outspoken critic of the climate lie, documents in his book *The Climate Chronicles* how weather patterns and events claimed to be evidence of climate change have been happening since long before industrialisation: 'What happened before naturally is happening again, as is to be expected given the cyclical nature of the climate due to the design of the planet.' If you read the detailed background to the climate hoax in my other books you will shake your head and wonder how anyone could believe the crap which has spawned a multi-trillion dollar industry based on absolute garbage (see HIV causes AIDs and Sars-Cov-2 causes 'Covid-19'). Climate and 'Covid' have much in common given they have the same source. They both have the contradictory *everything* factor in which everything is explained by reference to them. It's hot – 'it's climate change'. It's cold – 'it's climate change'. I got a sniffle – 'it's Covid'. I haven't got a sniffle – 'it's Covid'. Not having a sniffle has to be a symptom of 'Covid'. Everything is and not having a sniffle is especially dangerous if you are a slow walker. For sheer audacity I offer you a Cambridge University 'study' that actually linked 'Covid' to 'climate change'. It had to happen eventually. They concluded that climate change played a role in 'Covid-19' spreading from animals to humans because … wait for it … I kid you not … *the two groups were forced closer together as populations grow*. Er, that's it. The whole foundation on which this depended was that 'Bats are the likely zoonotic origin of SARS-CoV-1 and SARS-CoV-2'. Well, they are not. They are nothing to do with it. Apart from bats not being the origin and therefore 'climate change' effects on bats being irrelevant I am in awe of their academic insight. Where would we be without them? Not where we are that's for sure.

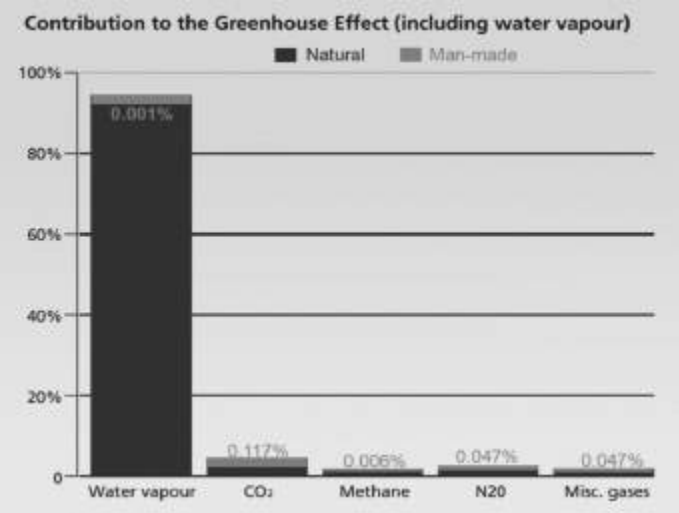


Figure 9: The idea that the gas of life is disastrously changing the climate is an insult to brain cell activity.

One other point about the weather is that climate modification is now well advanced and not every major weather event is natural – or earthquake come to that. I cover this subject at some length in other books. China is openly planning a rapid expansion of its weather modification programme which includes changing the climate in an area more than one and a half times the size of India. China used weather manipulation to ensure clear skies during the 2008 Olympics in Beijing. I have quoted from US military documents detailing how to employ weather manipulation as a weapon of war and they did that in the 1960s and 70s during the conflict in Vietnam with Operation Popeye manipulating monsoon rains for military purposes. Why would there be international treaties on weather modification if it wasn't possible? Of course it is. Weather is energetic information and it can be changed.

How was the climate hoax pulled off? See 'Covid'

If you can get billions to believe in a 'virus' that doesn't exist you can get them to believe in human-caused climate change that doesn't exist. Both are being used by the Cult to transform global society in the way it has long planned. Both hoaxes have been achieved in pretty much the same way. First you declare a lie is a fact. There's a

'virus' you call SARS-Cov-2 or humans are warming the planet with their behaviour. Next this becomes, via Cult networks, the foundation of government, academic and science policy and belief. Those who parrot the mantra are given big grants to produce research that confirms the narrative is true and ever more 'symptoms' are added to make the 'virus'/'climate change' sound even more scary. Scientists and researchers who challenge the narrative have their grants withdrawn and their careers destroyed. The media promote the lie as the unquestionable truth and censor those with an alternative view or evidence. A great percentage of the population believe what they are told as the lie becomes an everybody-knows-that and the believing-masses turn on those with a mind of their own. The technique has been used endlessly throughout human history. Wokers are the biggest promotors of the climate lie *and* 'Covid' fascism because their minds are owned by the Cult; their sense of self-righteous self-purity knows no bounds; and they exist in a bubble of reality in which facts are irrelevant and only get in the way of looking without seeing.

Running through all of this like veins in a blue cheese is control of information, which means control of perception, which means control of behaviour, which collectively means control of human society. The Cult owns the global media and Silicon Valley fascists for the simple reason that it *has* to. Without control of information it can't control perception and through that human society. Examine every facet of the Cult agenda and you will see that anything supporting its introduction is never censored while anything pushing back is always censored. I say again: Psychopaths that know why they are doing this must go before Nuremberg trials and those that follow their orders must trot along behind them into the same dock. 'I was just following orders' didn't work the first time and it must not work now. Nuremberg trials must be held all over the world before public juries for politicians, government officials, police, compliant doctors, scientists and virologists, and all Cult operatives such as Gates, Tedros, Fauci, Vallance, Whitty, Ferguson, Zuckerberg, Wojcicki, Brin, Page, Dorsey, the whole damn lot of

them – including, no *especially*, the psychopath psychologists. Without them and the brainless, gutless excuses for journalists that have repeated their lies, none of this could be happening. Nobody can be allowed to escape justice for the psychological and economic Armageddon they are all responsible for visiting upon the human race.

As for the compliant, unquestioning, swathes of humanity, and the self-obsessed, all-knowing ignorance of the Wokers … don't start me. God help their kids. God help their grandkids. God *help them*.

CHAPTER NINE

We must have it? So what is it?

Well I won't back down. No, I won't back down. You can stand me up at the Gates of Hell. But I won't back down
Tom Petty

I will now focus on the genetically-manipulating 'Covid vaccines' which do not meet this official definition of a vaccine by the US Centers for Disease Control (CDC): 'A product that stimulates a person's immune system to produce immunity to a specific disease, protecting the person from that disease.' On that basis 'Covid vaccines' are not a vaccine in that the makers don't even claim they stop infection or transmission.

They are instead part of a multi-levelled conspiracy to change the nature of the human body and what it means to be 'human' and to depopulate an enormous swathe of humanity. What I shall call Human 1.0 is on the cusp of becoming Human 2.0 and for very sinister reasons. Before I get to the 'Covid vaccine' in detail here's some background to vaccines in general. Government regulators do not test vaccines – the makers do – and the makers control which data is revealed and which isn't. Children in America are given 50 vaccine doses by age six and 69 by age 19 and the effect of the whole combined schedule has never been tested. Autoimmune diseases when the immune system attacks its own body have soared in the mass vaccine era and so has disease in general in children and the young. Why wouldn't this be the case when vaccines target the *immune system?* The US government gave Big Pharma drug

companies immunity from prosecution for vaccine death and injury in the 1986 National Childhood Vaccine Injury Act (NCVIA) and since then the government (taxpayer) has been funding compensation for the consequences of Big Pharma vaccines. The criminal and satanic drug giants can't lose and the vaccine schedule has increased dramatically since 1986 for this reason. There is no incentive to make vaccines safe and a big incentive to make money by introducing ever more. Even against a ridiculously high bar to prove vaccine liability, and with the government controlling the hearing in which it is being challenged for compensation, the vaccine court has so far paid out more than $4 billion. These are the vaccines we are told are safe and psychopaths like Zuckerberg censor posts saying otherwise. The immunity law was even justified by a ruling that vaccines by their nature were 'unavoidably unsafe'.

Check out the ingredients of vaccines and you will be shocked if you are new to this. *They put that in children's bodies?? What??* Try aluminium, a brain toxin connected to dementia, aborted foetal tissue and formaldehyde which is used to embalm corpses. World-renowned aluminium expert Christopher Exley had his research into the health effect of aluminium in vaccines shut down by Keele University in the UK when it began taking funding from the Bill and Melinda Gates Foundation. Research when diseases 'eradicated' by vaccines began to decline and you will find the fall began long *before* the vaccine was introduced. Sometimes the fall even plateaued after the vaccine. Diseases like scarlet fever for which there was no vaccine declined in the same way because of environmental and other factors. A perfect case in point is the polio vaccine. Polio began when lead arsenate was first sprayed as an insecticide and residues remained in food products. Spraying started in 1892 and the first US polio epidemic came in Vermont in 1894. The simple answer was to stop spraying, but Rockefeller-created Big Pharma had a better idea. Polio was decreed to be caused by the *poliovirus* which 'spreads from person to person and can infect a person's spinal cord'. Lead arsenate was replaced by the lethal DDT which had the same effect of causing paralysis by damaging the brain and central nervous

system. Polio plummeted when DDT was reduced and then banned, but the vaccine is still given the credit for something it didn't do. Today by far the biggest cause of polio is the vaccines promoted by Bill Gates. Vaccine justice campaigner Robert Kennedy Jr, son of assassinated (by the Cult) US Attorney General Robert Kennedy, wrote:

> In 2017, the World Health Organization (WHO) reluctantly admitted that the global explosion in polio is predominantly vaccine strain. The most frightening epidemics in Congo, Afghanistan, and the Philippines, are all linked to vaccines. In fact, by 2018, 70% of global polio cases were vaccine strain.

Vaccines make fortunes for Cult-owned Gates and Big Pharma while undermining the health and immune systems of the population. We had a glimpse of the mentality behind the Big Pharma cartel with a report on WION (World is One News), an international English language TV station based in India, which exposed the extraordinary behaviour of US drug company Pfizer over its 'Covid vaccine'. The WION report told how Pfizer had made fantastic demands of Argentina, Brazil and other countries in return for its 'vaccine'. These included immunity from prosecution, even for Pfizer negligence, government insurance to protect Pfizer from law suits and handing over as collateral sovereign assets of the country to include Argentina's bank reserves, military bases and embassy buildings. Pfizer demanded the same of Brazil in the form of waiving sovereignty of its assets abroad; exempting Pfizer from Brazilian laws; and giving Pfizer immunity from all civil liability. This is a 'vaccine' developed with government funding. Big Pharma is evil incarnate as a creation of the Cult and all must be handed tickets to Nuremberg.

Phantom 'vaccine' for a phantom 'disease'

I'll expose the 'Covid vaccine' fraud and then go on to the wider background of why the Cult has set out to 'vaccinate' every man, woman and child on the planet for an alleged 'new disease' with a survival rate of 99.77 percent (or more) even by the grotesquely-

manipulated figures of the World Health Organization and Johns Hopkins University. The 'infection' to 'death' ratio is 0.23 to 0.15 percent according to Stanford epidemiologist Dr John Ioannidis and while estimates vary the danger remains tiny. I say that if the truth be told the fake infection to fake death ratio is zero. Never mind all the evidence I have presented here and in *The Answer* that there is no 'virus' let us just focus for a moment on that death-rate figure of say 0.23 percent. The figure includes all those worldwide who have tested positive with a test not testing for the 'virus' and then died within 28 days or even longer of any other cause – *any other cause*. Now subtract all those illusory 'Covid' deaths on the global data sheets from the 0.23 percent. What do you think you would be left with? *Zero*. A vaccination has never been successfully developed for a so-called coronavirus. They have all failed at the animal testing stage when they caused hypersensitivity to what they were claiming to protect against and made the impact of a disease far worse. Cult-owned vaccine corporations got around that problem this time by bypassing animal trials, going straight to humans and making the length of the 'trials' before the public rollout as short as they could get away with. Normally it takes five to ten years or more to develop vaccines that still cause demonstrable harm to many people and that's without including the long-term effects that are never officially connected to the vaccination. 'Covid' non-vaccines have been officially produced and approved in a matter of months from a standing start and part of the reason is that (a) they were developed before the 'Covid' hoax began and (b) they are based on computer programs and not natural sources. Official non-trials were so short that government agencies gave *emergency*, not full, approval. 'Trials' were not even completed and full approval cannot be secured until they are. Public 'Covid vaccination' is actually a *continuation of the trial*. Drug company 'trials' are not scheduled to end until 2023 by which time a lot of people are going to be dead. Data on which government agencies gave this emergency approval was supplied by the Big Pharma corporations themselves in the form of Pfizer/BioNTech, AstraZeneca, Moderna, Johnson & Johnson, and

others, and this is the case with all vaccines. By its very nature *emergency* approval means drug companies do not have to prove that the 'vaccine' is 'safe and effective'. How could they with trials way short of complete? Government regulators only have to *believe* that they *could* be safe and effective. It is criminal manipulation to get products in circulation with no testing worth the name. Agencies giving that approval are infested with Big Pharma-connected place-people and they act in the interests of Big Pharma (the Cult) and not the public about whom they do not give a damn.

More human lab rats

'Covid vaccines' produced in record time by Pfizer/BioNTech and Moderna employ a technique *never approved before for use on humans*. They are known as mRNA 'vaccines' and inject a synthetic version of 'viral' mRNA or 'messenger RNA'. The key is in the term 'messenger'. The body works, or doesn't, on the basis of information messaging. Communications are constantly passing between and within the genetic system and the brain. Change those messages and you change the state of the body and even its very nature and you can change psychology and behaviour by the way the brain processes information. I think you are going to see significant changes in personality and perception of many people who have had the 'Covid vaccine' synthetic potions. Insider Aldous Huxley predicted the following in 1961 and mRNA 'vaccines' can be included in the term 'pharmacological methods':

> There will be, in the next generation or so, a pharmacological method of making people love their servitude, and producing dictatorship without tears, so to speak, producing a kind of painless concentration camp for entire societies, so that people will in fact have their own liberties taken away from them, but rather enjoy it, because they will be distracted from any desire to rebel by propaganda or brainwashing, or brainwashing enhanced by pharmacological methods. And this seems to be the final revolution.

Apologists claim that mRNA synthetic 'vaccines' don't change the DNA genetic blueprint because RNA does not affect DNA only the other way round. This is so disingenuous. A process called 'reverse

transcription' can convert RNA into DNA and be integrated into DNA in the cell nucleus. This was highlighted in December, 2020, by scientists at Harvard and Massachusetts Institute of Technology (MIT). Geneticists report that more than 40 percent of mammalian genomes results from reverse transcription. On the most basic level if messaging changes then that sequence must lead to changes in DNA which is receiving and transmitting those communications. How can introducing synthetic material into cells not change the cells where DNA is located? The process is known as transfection which is defined as 'a technique to insert foreign nucleic acid (DNA or RNA) into a cell, typically with the intention of altering the properties of the cell'. Researchers at the Sloan Kettering Institute in New York found that changes in messenger RNA can deactivate tumour-suppressing proteins and thereby promote cancer. This is what happens when you mess with messaging. 'Covid vaccine' maker Moderna was founded in 2010 by Canadian stem cell biologist Derrick J. Rossi after his breakthrough discovery in the field of transforming and reprogramming stem cells. These are neutral cells that can be programmed to become any cell including sperm cells. Moderna was therefore founded on the principle of genetic manipulation and has never produced any vaccine or drug before its genetically-manipulating synthetic 'Covid' shite. Look at the name – Mode-RNA or Modify-RNA. Another important point is that the US Supreme Court has ruled that genetically-modified DNA, or complementary DNA (cDNA) synthesized in the laboratory from messenger RNA, can be patented and owned. These psychopaths are doing this to the human body.

Cells replicate synthetic mRNA in the 'Covid vaccines' and in theory the body is tricked into making antigens which trigger antibodies to target the 'virus spike proteins' which as Dr Tom Cowan said have *never been seen*. Cut the crap and these 'vaccines' deliver *self-replicating* synthetic material to the cells with the effect of changing human DNA. The more of them you have the more that process is compounded while synthetic material is all the time self-replicating. 'Vaccine'-maker Moderna describes mRNA as 'like

software for the cell' and so they are messing with the body's software. What happens when you change the software in a computer? Everything changes. For this reason the Cult is preparing a production line of mRNA 'Covid vaccines' and a long list of excuses to use them as with all the 'variants' of a 'virus' never shown to exist. The plan is further to transfer the mRNA technique to other vaccines mostly given to children and young people. The cumulative consequences will be a transformation of human DNA through a constant infusion of synthetic genetic material which will kill many and change the rest. Now consider that governments that have given emergency approval for a vaccine that's not a vaccine; never been approved for humans before; had no testing worth the name; and the makers have been given immunity from prosecution for any deaths or adverse effects suffered by the public. The UK government awarded *permanent legal indemnity* to itself and its employees for harm done when a patient is being treated for 'Covid-19' or 'suspected Covid-19'. That is quite a thought when these are possible 'side-effects' from the 'vaccine' (they are not 'side', they are effects) listed by the US Food and Drug Administration:

Guillain-Barre syndrome; acute disseminated encephalomyelitis; transverse myelitis; encephalitis; myelitis; encephalomyelitis; meningoencephalitis; meningitis; encephalopathy; convulsions; seizures; stroke; narcolepsy; cataplexy; anaphylaxis; acute myocardial infarction (heart attack); myocarditis; pericarditis; autoimmune disease; death; implications for pregnancy, and birth outcomes; other acute demyelinating diseases; non anaphylactic allergy reactions; thrombocytopenia ; disseminated intravascular coagulation; venous thromboembolism; arthritis; arthralgia; joint pain; Kawasaki disease; multisystem inflammatory syndrome in children; vaccine enhanced disease. The latter is the way the 'vaccine' has the potential to make diseases far worse than they would otherwise be.

UK doctor and freedom campaigner Vernon Coleman described the conditions in this list as 'all unpleasant, most of them very serious, and you can't get more serious than death'. The thought that anyone at all has had the 'vaccine' in these circumstances is testament to the potential that humanity has for clueless, unquestioning, stupidity and for many that programmed stupidity has already been terminal.

An insider speaks

Dr Michael Yeadon is a former Vice President, head of research and Chief Scientific Adviser at vaccine giant Pfizer. Yeadon worked on the inside of Big Pharma, but that did not stop him becoming a vocal critic of 'Covid vaccines' and their potential for multiple harms, including infertility in women. By the spring of 2021 he went much further and even used the no, no, term 'conspiracy'. When you begin to see what is going on it is impossible not to do so. Yeadon spoke out in an interview with freedom campaigner James Delingpole and I mentioned earlier how he said that no one had samples of 'the virus'. He explained that the mRNA technique originated in the anti-cancer field and ways to turn on and off certain genes which could be advantageous if you wanted to stop cancer growing out of control. 'That's the origin of them. They are a very unusual application, really.' Yeadon said that treating a cancer patient with an aggressive procedure might be understandable if the alternative was dying, but it was quite another thing to use the same technique as a public health measure. Most people involved wouldn't catch the infectious agent you were vaccinating against and if they did they probably wouldn't die:

> If you are really using it as a public health measure you really want to as close as you can get to zero sides-effects … I find it odd that they chose techniques that were really cutting their teeth in the field of oncology and I'm worried that in using gene-based vaccines that have to be injected in the body and spread around the body, get taken up into some cells, and the regulators haven't quite told us which cells they get taken up into … you are going to be generating a wide range of responses … with multiple steps each of which could go well or badly.

I doubt the Cult intends it to go well. Yeadon said that you can put any gene you like into the body through the 'vaccine'. 'You can certainly give them a gene that would do them some harm if you wanted.' I was intrigued when he said that when used in the cancer field the technique could turn genes on and off. I explore this process in *The Answer* and with different genes having different functions you could create mayhem – physically and psychologically – if you turned the wrong ones on and the right ones off. I read reports of an experiment by researchers at the University of Washington's school of computer science and engineering in which they encoded DNA to infect computers. The body is itself a biological computer and if human DNA can inflict damage on a computer why can't the computer via synthetic material mess with the human body? It can. The Washington research team said it was possible to insert malicious malware into 'physical DNA strands' and corrupt the computer system of a gene sequencing machine as it 'reads gene letters and stores them as binary digits 0 and 1'. They concluded that hackers could one day use blood or spit samples to access computer systems and obtain sensitive data from police forensics labs or infect genome files. It is at this level of digital interaction that synthetic 'vaccines' need to be seen to get the full picture and that will become very clear later on. Michael Yeadon said it made no sense to give the 'vaccine' to younger people who were in no danger from the 'virus'. What was the benefit? It was all downside with potential effects:

> The fact that my government in what I thought was a civilised, rational country, is raining [the 'vaccine'] on people in their 30s and 40s, even my children in their 20s, they're getting letters and phone calls, I know this is not right and any of you doctors who are vaccinating you know it's not right, too. They are not at risk. They are not at risk from the disease, so you are now hoping that the side-effects are so rare that you get away with it. You don't give new technology … that you don't understand to 100 percent of the population.

Blood clot problems with the AstraZeneca 'vaccine' have been affecting younger people to emphasise the downside risks with no benefit. AstraZeneca's version, produced with Oxford University, does not use mRNA, but still gets its toxic cocktail inside cells where

it targets DNA. The Johnson & Johnson 'vaccine' which uses a similar technique has also produced blood clot effects to such an extent that the United States paused its use at one point. They are all 'gene therapy' (cell modification) procedures and not 'vaccines'. The truth is that once the content of these injections enter cells we have no idea what the effect will be. People can speculate and some can give very educated opinions and that's good. In the end, though, only the makers know what their potions are designed to do and even they won't know every last consequence. Michael Yeadon was scathing about doctors doing what they knew to be wrong. 'Everyone's mute', he said. Doctors in the NHS must know this was not right, coming into work and injecting people. 'I don't know how they sleep at night. I know I couldn't do it. I know that if I were in that position I'd have to quit.' He said he knew enough about toxicology to know this was not a good risk-benefit. Yeadon had spoken to seven or eight university professors and all except two would not speak out publicly. Their universities had a policy that no one said anything that countered the government and its medical advisors. They were afraid of losing their government grants. This is how intimidation has been used to silence the truth at every level of the system. I say silence, but these people could still speak out if they made that choice. Yeadon called them 'moral cowards' – 'This is about your children and grandchildren's lives and you have just buggered off and left it.'

'Variant' nonsense

Some of his most powerful comments related to the alleged 'variants' being used to instil more fear, justify more lockdowns, and introduce more 'vaccines'. He said government claims about 'variants' were nonsense. He had checked the alleged variant 'codes' and they were 99.7 percent identical to the 'original'. This was the human identity difference equivalent to putting a baseball cap on and off or wearing it the other way round. A 0.3 percent difference would make it impossible for that 'variant' to escape immunity from the 'original'. This made no sense of having new 'vaccines' for

'variants'. He said there would have to be at least a *30 percent* difference for that to be justified and even then he believed the immune system would still recognise what it was. Gates-funded 'variant modeller' and 'vaccine'-pusher John Edmunds might care to comment. Yeadon said drug companies were making new versions of the 'vaccine' as a 'top up' for 'variants'. Worse than that, he said, the 'regulators' around the world like the MHRA in the UK had got together and agreed that because 'vaccines' for 'variants' were so similar to the first 'vaccines' *they did not have to do safety studies*. How transparently sinister that is. This is when Yeadon said: 'There is a conspiracy here.' There was no need for another vaccine for 'variants' and yet we were told that there was and the country had shut its borders because of them. 'They are going into hundreds of millions of arms without passing 'go' or any regulator. Why did they do that? Why did they pick this method of making the vaccine?'

The reason had to be something bigger than that it seemed and 'it's not protection against the virus'. It's was a far bigger project that meant politicians and advisers were willing to do things and not do things that knowingly resulted in avoidable deaths – 'that's already happened when you think about lockdown and deprivation of health care for a year.' He spoke of people prepared to do something that results in the avoidable death of their fellow human beings and it not bother them. This is the penny-drop I have been working to get across for more than 30 years – the level of pure evil we are dealing with. Yeadon said his friends and associates could not believe there could be that much evil, but he reminded them of Stalin, Pol Pot and Hitler and of what Stalin had said: 'One death is a tragedy. A million? A statistic.' He could not think of a benign explanation for why you need top-up vaccines 'which I'm sure you don't' and for the regulators 'to just get out of the way and wave them through'. Why would the regulators do that when they were still wrestling with the dangers of the 'parent' vaccine? He was clearly shocked by what he had seen since the 'Covid' hoax began and now he was thinking the previously unthinkable:

If you wanted to depopulate a significant proportion of the world and to do it in a way that doesn't involve destruction of the environment with nuclear weapons, poisoning everyone with anthrax or something like that, and you wanted plausible deniability while you had a multi-year infectious disease crisis, I actually don't think you could come up with a better plan of work than seems to be in front of me. I can't say that's what they are going to do, but I can't think of a benign explanation why they are doing it.

He said he never thought that they would get rid of 99 percent of humans, but now he wondered. 'If you wanted to that this would be a hell of a way to do it – it would be unstoppable folks.' Yeadon had concluded that those who submitted to the 'vaccine' would be allowed to have some kind of normal life (but for how long?) while screws were tightened to coerce and mandate the last few percent. 'I think they'll put the rest of them in a prison camp. I wish I was wrong, but I don't think I am.' Other points he made included: There were no coronavirus vaccines then suddenly they all come along at the same time; we have no idea of the long term affect with trials so short; coercing or forcing people to have medical procedures is against the Nuremberg Code instigated when the Nazis did just that; people should at least delay having the 'vaccine'; a quick Internet search confirms that masks don't reduce respiratory viral transmission and 'the government knows that'; they have smashed civil society and they know that, too; two dozen peer-reviewed studies show no connection between lockdown and reducing deaths; he knew from personal friends the elite were still flying around and going on holiday while the public were locked down; the elite were not having the 'vaccines'. He was also asked if 'vaccines' could be made to target difference races. He said he didn't know, but the document by the Project for the New American Century in September, 2000, said developing 'advanced forms of biological warfare that can target *specific genotypes* may transform biological warfare from the realm of terror to a politically useful tool.' Oh, they're evil all right. Of that we can be *absolutely* sure.

Another cull of old people

We have seen from the CDC definition that the mRNA 'Covid vaccine' is not a vaccine and nor are the others that *claim* to reduce 'severity of symptoms' in *some* people, but not protect from infection or transmission. What about all the lies about returning to 'normal' if people were 'vaccinated'? If they are not claimed to stop infection and transmission of the alleged 'virus', how does anything change? This was all lies to manipulate people to take the jabs and we are seeing that now with masks and distancing still required for the 'vaccinated'. How did they think that elderly people with fragile health and immune responses were going to be affected by infusing their cells with synthetic material and other toxic substances? They *knew* that in the short and long term it would be devastating and fatal as the culling of the old that began with the first lockdowns was continued with the 'vaccine'. Death rates in care homes soared immediately residents began to be 'vaccinated' – infused with synthetic material. Brave and committed whistleblower nurses put their careers at risk by exposing this truth while the rest kept their heads down and their mouths shut to put their careers before those they are supposed to care for. A long-time American Certified Nursing Assistant who gave his name as James posted a video in which he described emotionally what happened in his care home when vaccination began. He said that during 2020 very few residents were sick with 'Covid' and no one died during the entire year; but shortly after the Pfizer mRNA injections 14 people died within two weeks and many others were near death. 'They're dropping like flies', he said. Residents who walked on their own before the shot could no longer and they had lost their ability to conduct an intelligent conversation. The home's management said the sudden deaths were caused by a 'super-spreader' of 'Covid-19'. Then how come, James asked, that residents who refused to take the injections were not sick? It was a case of inject the elderly with mRNA synthetic potions and blame their illness and death that followed on the 'virus'. James described what was happening in care homes as 'the greatest crime of genocide this country has ever seen'. Remember the NHS staff nurse from earlier who used the same

word ‘genocide’ for what was happening with the ‘vaccines’ and that it was an ‘act of human annihilation’. A UK care home whistleblower told a similar story to James about the effect of the ‘vaccine’ in deaths and ‘outbreaks’ of illness dubbed ‘Covid’ after getting the jab. She told how her care home management and staff had zealously imposed government regulations and no one was allowed to even question the official narrative let alone speak out against it. She said the NHS was even worse. Again we see the results of reframing. A worker at a local care home where I live said they had not had a single case of ‘Covid’ there for almost a year and when the residents were ‘vaccinated’ they had 19 positive cases in two weeks with eight dying.

It’s not the ‘vaccine’ – honest

The obvious cause and effect was being ignored by the media and most of the public. Australia’s health minister Greg Hunt (a former head of strategy at the World Economic Forum) was admitted to hospital after he had the ‘vaccine’. He was suffering according to reports from the skin infection ‘cellulitis’ and it must have been a severe case to have warranted days in hospital. Immediately the authorities said this was nothing to do with the ‘vaccine’ when an effect of some vaccines is a ‘cellulitis-like reaction’. We had families of perfectly healthy old people who died after the ‘vaccine’ saying that if only they had been given the ‘vaccine’ earlier they would still be alive. As a numbskull rating that is off the chart. A father of four ‘died of Covid’ at aged 48 when he was taken ill two days after having the ‘vaccine’. The man, a health administrator, had been ‘shielding during the pandemic’ and had ‘not really left the house’ until he went for the ‘vaccine’. Having the ‘vaccine’ and then falling ill and dying does not seem to have qualified as a possible cause and effect and ‘Covid-19’ went on his death certificate. His family said they had no idea how he ‘caught the virus’. A family member said: ‘Tragically, it could be that going for a vaccination ultimately led to him catching Covid …The sad truth is that they are never going to know where it came from.’ The family warned people to remember

that the virus still existed and was 'very real'. So was their stupidity. Nurses and doctors who had the first round of the 'vaccine' were collapsing, dying and ending up in a hospital bed while they or their grieving relatives were saying they'd still have the 'vaccine' again despite what happened. I kid you not. You mean if your husband returned from the dead he'd have the same 'vaccine' again that killed him??

Doctors at the VCU Medical Center in Richmond, Virginia, said the Johnson & Johnson 'vaccine' was to blame for a man's skin peeling off. Patient Richard Terrell said: 'It all just happened so fast. My skin peeled off. It's still coming off on my hands now.' He said it was stinging, burning and itching and when he bent his arms and legs it was very painful with 'the skin swollen and rubbing against itself'. Pfizer/BioNTech and Moderna vaccines use mRNA to change the cell while the Johnson & Johnson version uses DNA in a process similar to AstraZeneca's technique. Johnson & Johnson and AstraZeneca have both had their 'vaccines' paused by many countries after causing serious blood problems. Terrell's doctor Fnu Nutan said he could have died if he hadn't got medical attention. It sounds terrible so what did Nutan and Terrell say about the 'vaccine' now? Oh, they still recommend that people have it. A nurse in a hospital bed 40 minutes after the vaccination and unable to swallow due to throat swelling was told by a doctor that he lost mobility in his arm for 36 hours following the vaccination. What did he say to the ailing nurse? 'Good for you for getting the vaccination.' We are dealing with a serious form of cognitive dissonance madness in both public and medical staff. There is a remarkable correlation between those having the 'vaccine' and trumpeting the fact and suffering bad happenings shortly afterwards. Witold Rogiewicz, a Polish doctor, made a video of his 'vaccination' and ridiculed those who were questioning its safety and the intentions of Bill Gates: 'Vaccinate yourself to protect yourself, your loved ones, friends and also patients. And to mention quickly I have info for anti-vaxxers and anti-Coviders if you want to contact Bill Gates you can do this through me.' He further ridiculed the dangers of 5G. Days later he

was dead, but naturally the vaccination wasn't mentioned in the verdict of 'heart attack'.

Lies, lies and more lies

So many members of the human race have slipped into extreme states of insanity and unfortunately they include reframed doctors and nursing staff. Having a 'vaccine' and dying within minutes or hours is not considered a valid connection while death from any cause within 28 days or longer of a positive test with a test not testing for the 'virus' means 'Covid-19' goes on the death certificate. How could that 'vaccine'-death connection not have been made except by calculated deceit? US figures in the initial rollout period to February 12th, 2020, revealed that a third of the deaths reported to the CDC after 'Covid vaccines' happened within 48 hours. Five men in the UK suffered an 'extremely rare' blood clot problem after having the AstraZeneca 'vaccine', but no causal link was established said the Gates-funded Medicines and Healthcare products Regulatory Agency (MHRA) which had given the 'vaccine' emergency approval to be used. Former Pfizer executive Dr Michael Yeadon explained in his interview how the procedures could cause blood coagulation and clots. People who should have been at no risk were dying from blood clots in the brain and he said he had heard from medical doctor friends that people were suffering from skin bleeding and massive headaches. The AstraZeneca 'shot' was stopped by some 20 countries over the blood clotting issue and still the corrupt MHRA, the European Medicines Agency (EMA) and the World Health Organization said that it should continue to be given even though the EMA admitted that it 'still cannot rule out definitively' a link between blood clotting and the 'vaccine'. Later Marco Cavaleri, head of EMA vaccine strategy, said there was indeed a clear link between the 'vaccine' and thrombosis, but they didn't know why. So much for the trials showing the 'vaccine' is safe. Blood clots were affecting younger people who would be under virtually no danger from 'Covid' even if it existed which makes it all the more stupid and sinister.

The British government responded to public alarm by wheeling out June Raine, the terrifyingly weak infant school headmistress sound-alike who heads the UK MHRA drug 'regulator'. The idea that she would stand up to Big Pharma and government pressure is laughable and she told us that all was well in the same way that she did when allowing untested, never-used-on-humans-before, genetically-manipulating 'vaccines' to be exposed to the public in the first place. Mass lying is the new normal of the 'Covid' era. The MHRA later said 30 cases of rare blood clots had by then been connected with the AstraZeneca 'vaccine' (that means a lot more in reality) while stressing that the benefits of the jab in preventing 'Covid-19' outweighed any risks. A more ridiculous and disingenuous statement with callous disregard for human health it is hard to contemplate. Immediately after the mendacious 'all-clears' two hospital workers in Denmark experienced blood clots and cerebral haemorrhaging following the AstraZeneca jab and one died. Top Norwegian health official Pål Andre Holme said the 'vaccine' was the only common factor: 'There is nothing in the patient history of these individuals that can give such a powerful immune response … I am confident that the antibodies that we have found are the cause, and I see no other explanation than it being the vaccine which triggers it.' Strokes, a clot or bleed in the brain, were clearly associated with the 'vaccine' from word of mouth and whistleblower reports. Similar consequences followed with all these 'vaccines' that we were told were so safe and as the numbers grew by the day it was clear we were witnessing human carnage.

Learning the hard way

A woman interviewed by UKColumn told how her husband suffered dramatic health effects after the vaccine when he'd been in good health all his life. He went from being a little unwell to losing all feeling in his legs and experiencing 'excruciating pain'. Misdiagnosis followed twice at Accident and Emergency (an 'allergy' and 'sciatica') before he was admitted to a neurology ward where doctors said his serious condition had been caused by the

'vaccine'. Another seven 'vaccinated' people were apparently being treated on the same ward for similar symptoms. The woman said he had the 'vaccine' because they believed media claims that it was safe. 'I didn't think the government would give out a vaccine that does this to somebody; I believed they would be bringing out a vaccination that would be safe.' What a tragic way to learn that lesson. Another woman posted that her husband was transporting stroke patients to hospital on almost every shift and when he asked them if they had been 'vaccinated' for 'Covid' they all replied 'yes'. One had a 'massive brain bleed' the day after his second dose. She said her husband reported the 'just been vaccinated' information every time to doctors in A and E only for them to ignore it, make no notes and appear annoyed that it was even mentioned. This particular report cannot be verified, but it expresses a common theme that confirms the monumental underreporting of 'vaccine' consequences. Interestingly as the 'vaccines' and their brain blood clot/stroke consequences began to emerge the UK National Health Service began a publicity campaign telling the public what to do in the event of a stroke. A Scottish NHS staff nurse who quit in disgust in March, 2021, said:

I have seen traumatic injuries from the vaccine, they're not getting reported to the yellow card [adverse reaction] scheme, they're treating the symptoms, not asking why, why it's happening. It's just treating the symptoms and when you speak about it you're dismissed like you're crazy, I'm not crazy, I'm not crazy because every other colleague I've spoken to is terrified to speak out, they've had enough.

Videos appeared on the Internet of people uncontrollably shaking after the 'vaccine' with no control over muscles, limbs and even their face. A Scottish mother broke out in a severe rash all over her body almost immediately after she was given the AstraZeneca 'vaccine'. The pictures were horrific. Leigh King, a 41-year-old hairdresser from Lanarkshire said: 'Never in my life was I prepared for what I was about to experience … My skin was so sore and constantly hot … I have never felt pain like this …' But don't you worry, the 'vaccine' is perfectly safe. Then there has been the effect on medical

staff who have been pressured to have the 'vaccine' by psychopathic 'health' authorities and government. A London hospital consultant who gave the name K. Polyakova wrote this to the *British Medical Journal* or *BMJ*:

> I am currently struggling with … the failure to report the reality of the morbidity caused by our current vaccination program within the health service and staff population. The levels of sickness after vaccination is unprecedented and staff are getting very sick and some with neurological symptoms which is having a huge impact on the health service function. Even the young and healthy are off for days, some for weeks, and some requiring medical treatment. Whole teams are being taken out as they went to get vaccinated together.
>
> Mandatory vaccination in this instance is stupid, unethical and irresponsible when it comes to protecting our staff and public health. We are in the voluntary phase of vaccination, and encouraging staff to take an unlicensed product that is impacting on their immediate health … it is clearly stated that these vaccine products do not offer immunity or stop transmission. In which case why are we doing it?

Not to protect health that's for sure. Medical workers are lauded by governments for agenda reasons when they couldn't give a toss about them any more than they can for the population in general. Schools across America faced the same situation as they closed due to the high number of teachers and other staff with bad reactions to the Pfizer/BioNTech, Moderna, and Johnson & Johnson 'Covid vaccines' all of which were linked to death and serious adverse effects. The *BMJ* took down the consultant's comments pretty quickly on the grounds that they were being used to spread 'disinformation'. They were exposing the truth about the 'vaccine' was the real reason. The cover-up is breathtaking.

Hiding the evidence

The scale of the 'vaccine' death cover-up worldwide can be confirmed by comparing official figures with the personal experience of the public. I heard of many people in my community who died immediately or soon after the vaccine that would never appear in the media or even likely on the official totals of 'vaccine' fatalities and adverse reactions when only about ten percent are estimated to be

reported and I have seen some estimates as low as one percent in a Harvard study. In the UK alone by April 29th, 2021, some 757,654 adverse reactions had been officially reported from the Pfizer/BioNTech, Oxford/AstraZeneca and Moderna 'vaccines' with more than a thousand deaths linked to jabs and that means an estimated ten times this number in reality from a ten percent reporting rate percentage. That's seven million adverse reactions and 10,000 potential deaths and a one percent reporting rate would be ten times *those* figures. In 1976 the US government pulled the swine flu vaccine after *53* deaths. The UK data included a combined 10,000 eye disorders from the 'Covid vaccines' with more than 750 suffering visual impairment or blindness and again multiply by the estimated reporting percentages. As 'Covid cases' officially fell hospitals virtually empty during the 'Covid crisis' began to fill up with a range of other problems in the wake of the 'vaccine' rollout. The numbers across America have also been catastrophic. Deaths linked to *all* types of vaccine increased by *6,000 percent* in the first quarter of 2021 compared with 2020. A 39-year-old woman from Ogden, Utah, died four days after receiving a second dose of Moderna's 'Covid vaccine' when her liver, heart and kidneys all failed despite the fact that she had no known medical issues or conditions. Her family sought an autopsy, but Dr Erik Christensen, Utah's chief medical examiner, said proving vaccine injury as a cause of death almost never happened. He could think of only one instance where an autopsy would name a vaccine as the official cause of death and that would be anaphylaxis where someone received a vaccine and died almost instantaneously. 'Short of that, it would be difficult for us to definitively say this is the vaccine,' Christensen said. If that is true this must be added to the estimated ten percent (or far less) reporting rate of vaccine deaths and serious reactions and the conclusion can only be that vaccine deaths and serious reactions – including these 'Covid' potions' – are phenomenally understated in official figures. The same story can be found everywhere. Endless accounts of deaths and serious reactions among the public, medical

and care home staff while official figures did not even begin to reflect this.

Professional script-reader Dr David Williams, a 'top public-health official' in Ontario, Canada, insulted our intelligence by claiming only four serious adverse reactions and no deaths from the more than 380,000 vaccine doses then given. This bore no resemblance to what people knew had happened in their owns circles and we had Dirk Huyer in charge of getting millions vaccinated in Ontario while at the same time he was Chief Coroner for the province investigating causes of death including possible death from the vaccine. An aide said he had stepped back from investigating deaths, but evidence indicated otherwise. Rosemary Frei, who secured a Master of Science degree in molecular biology at the Faculty of Medicine at Canada's University of Calgary before turning to investigative journalism, was one who could see that official figures for 'vaccine' deaths and reactions made no sense. She said that doctors seldom reported adverse events and when people got really sick or died after getting a vaccination they would attribute that to anything except the vaccines. It had been that way for years and anyone who wondered aloud whether the 'Covid vaccines' or other shots cause harm is immediately branded as 'anti-vax' and 'anti-science'. This was 'career-threatening' for health professionals. Then there was the huge pressure to support the push to 'vaccinate' billions in the quickest time possible. Frei said:

> So that's where we're at today. More than half a million vaccine doses have been given to people in Ontario alone. The rush is on to vaccinate all 15 million of us in the province by September. And the mainstream media are screaming for this to be sped up even more. That all adds up to only a very slim likelihood that we're going to be told the truth by officials about how many people are getting sick or dying from the vaccines.

What is true of Ontario is true of everywhere.

They KNEW – and still did it

The authorities knew what was going to happen with multiple deaths and adverse reactions. The UK government's Gates-funded

and Big Pharma-dominated Medicines and Healthcare products Regulatory Agency (MHRA) hired a company to employ AI in compiling the projected reactions to the 'vaccine' that would otherwise be uncountable. The request for applications said: 'The MHRA urgently seeks an Artificial Intelligence (AI) software tool to process the expected high volume of Covid-19 vaccine Adverse Drug Reaction …' This was from the agency, headed by the disingenuous June Raine, that gave the 'vaccines' emergency approval and the company was hired before the first shot was given. 'We are going to kill and maim you – is that okay?' 'Oh, yes, perfectly fine – I'm very grateful, thank you, doctor.' The range of 'Covid vaccine' adverse reactions goes on for page after page in the MHRA criminally underreported 'Yellow Card' system and includes affects to eyes, ears, skin, digestion, blood and so on. Raine's MHRA amazingly claimed that the 'overall safety experience … is so far as expected from the clinical trials'. The death, serious adverse effects, deafness and blindness were *expected?* When did they ever mention that? If these human tragedies were expected then those that gave approval for the use of these 'vaccines' must be guilty of crimes against humanity including murder – a definition of which is 'killing a person with malice aforethought or with recklessness manifesting extreme indifference to the value of human life.' People involved at the MHRA, the CDC in America and their equivalent around the world must go before Nuremberg trials to answer for their callous inhumanity. We are only talking here about the immediate effects of the 'vaccine'. The longer-term impact of the DNA synthetic manipulation is the main reason they are so hysterically desperate to inoculate the entire global population in the shortest possible time.

Africa and the developing world are a major focus for the 'vaccine' depopulation agenda and a mass vaccination sales-pitch is underway thanks to caring people like the Rockefellers and other Cult assets. The Rockefeller Foundation, which pre-empted the 'Covid pandemic' in a document published in 2010 that 'predicted' what happened a decade later, announced an initial $34.95 million grant in February, 2021, 'to ensure more equitable access to Covid-19

testing and vaccines' among other things in Africa in collaboration with '24 organizations, businesses, and government agencies'. The pan-Africa initiative would focus on 10 countries: Burkina Faso, Ethiopia, Ghana, Kenya, Nigeria, Rwanda, South Africa, Tanzania, Uganda, and Zambia'. Rajiv Shah, President of the Rockefeller Foundation and former administrator of CIA-controlled USAID, said that if Africa was not mass-vaccinated (to change the DNA of its people) it was a 'threat to all of humanity' and not fair on Africans. When someone from the Rockefeller Foundation says they want to do something to help poor and deprived people and countries it is time for a belly-laugh. They are doing this out of the goodness of their 'heart' because 'vaccinating' the entire global population is what the 'Covid' hoax set out to achieve. Official 'decolonisation' of Africa by the Cult was merely a prelude to financial colonisation on the road to a return to physical colonisation. The 'vaccine' is vital to that and the sudden and convenient death of the 'Covid' sceptic president of Tanzania can be seen in its true light. A lot of people in Africa are aware that this is another form of colonisation and exploitation and they need to stand their ground.

The 'vaccine is working' scam

A potential problem for the Cult was that the 'vaccine' is meant to change human DNA and body messaging and not to protect anyone from a 'virus' never shown to exist. The vaccine couldn't work because it was not designed to work and how could they make it *appear* to be working so that more people would have it? This was overcome by lowering the amplification rate of the PCR test to produce fewer 'cases' and therefore fewer 'deaths'. Some of us had been pointing out since March, 2020, that the amplification rate of the test not testing for the 'virus' had been made artificially high to generate positive tests which they could call 'cases' to justify lockdowns. The World Health Organization recommended an absurdly high 45 amplification cycles to ensure the high positives required by the Cult and then remained silent on the issue until January 20th, 2021 – Biden's Inauguration Day. This was when the

'vaccinations' were seriously underway and on that day the WHO recommended after discussions with America's CDC that laboratories *lowered their testing amplification*. Dr David Samadi, a certified urologist and health writer, said the WHO was encouraging all labs to reduce their cycle count for PCR tests. He said the current cycle was much too high and was 'resulting in any particle being declared a positive case'. Even one mainstream news report I saw said this meant the number of 'Covid' infections may have been 'dramatically inflated'. Oh, just a little bit. The CDC in America issued new guidance to laboratories in April, 2021, to use 28 cycles *but only for 'vaccinated' people*. The timing of the CDC/WHO interventions were cynically designed to make it appear the 'vaccines' were responsible for falling cases and deaths when the real reason can be seen in the following examples. New York's state lab, the Wadsworth Center, identified 872 positive tests in July, 2020, based on a threshold of 40 cycles. When the figure was lowered to 35 cycles *43 percent* of the 872 were no longer 'positives'. At 30 cycles the figure was 63 percent. A Massachusetts lab found that between *85 to 90 percent* of people who tested positive in July with a cycle threshold of 40 would be negative at 30 cycles, Ashish Jha, MD, director of the Harvard Global Health Institute, said: 'I'm really shocked that it could be that high … Boy, does it really change the way we need to be thinking about testing.' I'm shocked that I could see the obvious in the spring of 2020, with no medical background, and most medical professionals still haven't worked it out. No, that's not shocking – it's terrifying.

Three weeks after the WHO directive to lower PCR cycles the London *Daily Mail* ran this headline: 'Why ARE Covid cases plummeting? New infections have fallen 45% in the US and 30% globally in the past 3 weeks but experts say vaccine is NOT the main driver because only 8% of Americans and 13% of people worldwide have received their first dose.' They acknowledged that the drop could not be attributed to the 'vaccine', but soon this morphed throughout the media into the 'vaccine' has caused cases and deaths to fall when it was the PCR threshold. In December, 2020, there was

chaos at English Channel ports with truck drivers needing negative 'Covid' tests before they could board a ferry home for Christmas. The government wanted to remove the backlog as fast as possible and they brought in troops to do the 'testing'. Out of 1,600 drivers just *36* tested positive and the rest were given the all clear to cross the Channel. I guess the authorities thought that 36 was the least they could get away with without the unquestioning catching on. The amplification trick which most people believed in the absence of information in the mainstream applied more pressure on those refusing the 'vaccine' to succumb when it 'obviously worked'. The truth was the exact opposite with deaths in care homes soaring with the 'vaccine' and in Israel the term used was 'skyrocket'. A re-analysis of published data from the Israeli Health Ministry led by Dr Hervé Seligmann at the Medicine Emerging Infectious and Tropical Diseases at Aix-Marseille University found that Pfizer's 'Covid vaccine' killed 'about 40 times more [elderly] people than the disease itself would have killed' during a five-week vaccination period and *260 times* more younger people than would have died from the 'virus' even according to the manipulated 'virus' figures. Dr Seligmann and his co-study author, Haim Yativ, declared after reviewing the Israeli 'vaccine' death data: 'This is a new Holocaust.'

Then, in mid-April, 2021, after vast numbers of people worldwide had been 'vaccinated', the story changed with clear coordination. The UK government began to prepare the ground for more future lockdowns when Nuremberg-destined Boris Johnson told yet another whopper. He said that cases had fallen because of *lockdowns* not 'vaccines'. Lockdowns are irrelevant when *there is no 'virus'* and the test and fraudulent death certificates are deciding the number of 'cases' and 'deaths'. Study after study has shown that lockdowns don't work and instead kill and psychologically destroy people. Meanwhile in the United States Anthony Fauci and Rochelle Walensky, the ultra-Zionist head of the CDC, peddled the same line. More lockdown was the answer and not the 'vaccine', a line repeated on cue by the moron that is Canadian Prime Minister Justin Trudeau. Why all the hysteria to get everyone 'vaccinated' if lockdowns and

not 'vaccines' made the difference? None of it makes sense on the face of it. Oh, but it does. The Cult wants lockdowns *and* the 'vaccine' and if the 'vaccine' is allowed to be seen as the total answer lockdowns would no longer be justified when there are still livelihoods to destroy. 'Variants' and renewed upward manipulation of PCR amplification are planned to instigate never-ending lockdown *and* more 'vaccines'.

You *must* have it – we're desperate

Israel, where the Jewish and Arab population are ruled by the Sabbatian Cult, was the front-runner in imposing the DNA-manipulating 'vaccine' on its people to such an extent that Jewish refusers began to liken what was happening to the early years of Nazi Germany. This would seem to be a fantastic claim. Why would a government of Jewish people be acting like the Nazis did? If you realise that the Sabbatian Cult was behind the Nazis and that Sabbatians hate Jews the pieces start to fit and the question of why a 'Jewish' government would treat Jews with such callous disregard for their lives and freedom finds an answer. Those controlling the government of Israel *aren't Jewish* – they're Sabbatian. Israeli lawyer Tamir Turgal was one who made the Nazi comparison in comments to German lawyer Reiner Fuellmich who is leading a class action lawsuit against the psychopaths for crimes against humanity. Turgal described how the Israeli government was vaccinating children and pregnant women on the basis that there was no evidence that this was dangerous when they had no evidence that it *wasn't* dangerous either. They just had no evidence. This was medical experimentation and Turgal said this breached the Nuremberg Code about medical experimentation and procedures requiring informed consent and choice. Think about that. A Nuremberg Code developed because of Nazi experimentation on Jews and others in concentration camps by people like the evil-beyond-belief Josef Mengele is being breached by the *Israeli* government; but when you know that it's a *Sabbatian* government along with its intelligence and military agencies like Mossad, Shin Bet and the Israeli Defense Forces, and that Sabbatians

were the force behind the Nazis, the kaleidoscope comes into focus. What have we come to when Israeli Jews are suing their government for violating the Nuremberg Code by essentially making Israelis subject to a medical experiment using the controversial 'vaccines'? It's a shocker that this has to be done in the light of what happened in Nazi Germany. The Anshe Ha-Emet, or 'People of the Truth', made up of Israeli doctors, lawyers, campaigners and public, have launched a lawsuit with the International Criminal Court. It says:

> When the heads of the Ministry of Health as well as the prime minister presented the vaccine in Israel and began the vaccination of Israeli residents, the vaccinated were not advised, that, in practice, they are taking part in a medical experiment and that their consent is required for this under the Nuremberg Code.

The irony is unbelievable, but easily explained in one word: Sabbatians. The foundation of Israeli 'Covid' apartheid is the 'green pass' or 'green passport' which allows Jews and Arabs who have had the DNA-manipulating 'vaccine' to go about their lives – to work, fly, travel in general, go to shopping malls, bars, restaurants, hotels, concerts, gyms, swimming pools, theatres and sports venues, while non-'vaccinated' are banned from all those places and activities. Israelis have likened the 'green pass' to the yellow stars that Jews in Nazi Germany were forced to wear – the same as the yellow stickers that a branch of UK supermarket chain Morrisons told exempt mask-wears they had to display when shopping. How very sensitive. The Israeli system is blatant South African-style apartheid on the basis of compliance or non-compliance to fascism rather than colour of the skin. How appropriate that the Sabbatian Israeli government was so close to the pre-Mandela apartheid regime in Pretoria. The Sabbatian-instigated 'vaccine passport' in Israel is planned for everywhere. Sabbatians struck a deal with Pfizer that allowed them to lead the way in the percentage of a national population infused with synthetic material and the result was catastrophic. Israeli freedom activist Shai Dannon told me how chairs were appearing on beaches that said 'vaccinated only'. Health Minister Yuli Edelstein said that anyone unwilling or unable to get

the jabs that 'confer immunity' will be 'left behind'. The man's a liar. Not even the makers claim the 'vaccines' confer immunity. When you see those figures of 'vaccine' deaths these psychopaths were saying that you must take the chance the 'vaccine' will kill you or maim you while knowing it will change your DNA or lockdown for you will be permanent. That's fascism. The Israeli parliament passed a law to allow personal information of the non-vaccinated to be shared with local and national authorities for three months. This was claimed by its supporters to be a way to 'encourage' people to be vaccinated. Hadas Ziv from Physicians for Human Rights described this as a 'draconian law which crushed medical ethics and the patient rights'. But that's the idea, the Sabbatians would reply.

Your papers, please

Sabbatian Israel was leading what has been planned all along to be a global 'vaccine pass' called a 'green passport' without which you would remain in permanent lockdown restriction and unable to do anything. This is how badly – *desperately* – the Cult is to get everyone 'vaccinated'. The term and colour 'green' was not by chance and related to the psychology of fusing the perception of the green climate hoax with the 'Covid' hoax and how the 'solution' to both is the same Great Reset. Lying politicians, health officials and psychologists denied there were any plans for mandatory vaccinations or restrictions based on vaccinations, but they knew that was exactly what was meant to happen with governments of all countries reaching agreements to enforce a global system. 'Free' Denmark and 'free' Sweden unveiled digital vaccine certification. Cyprus, Czech Republic, Estonia, Greece, Hungary, Iceland, Italy, Poland, Portugal, Slovakia, and Spain have all committed to a vaccine passport system and the rest including the whole of the EU would follow. The satanic UK government will certainly go this way despite mendacious denials and at the time of writing it is trying to manipulate the public into having the 'vaccine' so they could go abroad on a summer holiday. How would that work without something to prove you had the synthetic toxicity injected into you?

Documents show that the EU's European Commission was moving towards 'vaccine certificates' in 2018 and 2019 before the 'Covid' hoax began. They knew what was coming. Abracadabra – Ursula von der Leyen, the German President of the Commission, announced in March, 2021, an EU 'Digital Green Certificate' – green again – to track the public's 'Covid status'. The passport sting is worldwide and the Far East followed the same pattern with South Korea ruling that only those with 'vaccination' passports – again the *green* pass – would be able to 'return to their daily lives'.

Bill Gates has been preparing for this 'passport' with other Cult operatives for years and beyond the paper version is a Gates-funded 'digital tattoo' to identify who has been vaccinated and who hasn't. The 'tattoo' is reported to include a substance which is externally readable to confirm who has been vaccinated. This is a bio-luminous light-generating enzyme (think fireflies) called … *Luciferase*. Yes, named after the Cult 'god' Lucifer the 'light bringer' of whom more to come. Gates said he funded the readable tattoo to ensure children in the developing world were vaccinated and no one was missed out. He cares so much about poor kids as we know. This was just the cover story to develop a vaccine tagging system for everyone on the planet. Gates has been funding the ID2020 'alliance' to do just that in league with other lovely people at Microsoft, GAVI, the Rockefeller Foundation, Accenture and IDEO.org. He said in interviews in March, 2020, before any 'vaccine' publicly existed, that the world must have a globalised digital certificate to track the 'virus' and who had been vaccinated. Gates knew from the start that the mRNA vaccines were coming and when they would come and that the plan was to tag the 'vaccinated' to marginalise the intelligent and stop them doing anything including travel. Evil just doesn't suffice. Gates was exposed for offering a $10 million bribe to the Nigerian House of Representatives to invoke compulsory 'Covid' vaccination of all Nigerians. Sara Cunial, a member of the Italian Parliament, called Gates a 'vaccine criminal'. She urged the Italian President to hand him over to the International Criminal Court for crimes against

humanity and condemned his plans to 'chip the human race' through ID2020.

You know it's a long-planned agenda when war criminal and Cult gofer Tony Blair is on the case. With the scale of arrogance only someone as dark as Blair can muster he said: 'Vaccination in the end is going to be your route to liberty.' Blair is a disgusting piece of work and he confirms that again. The media has given a lot of coverage to a bloke called Charlie Mullins, founder of London's biggest independent plumbing company, Pimlico Plumbers, who has said he won't employ anyone who has not been vaccinated or have them go to any home where people are not vaccinated. He said that if he had his way no one would be allowed to walk the streets if they have not been vaccinated. Gates was cheering at the time while I was alerting the white coats. The plan is that people will qualify for 'passports' for having the first two doses and then to keep it they will have to have all the follow ups and new ones for invented 'variants' until human genetics is transformed and many are dead who can't adjust to the changes. Hollywood celebrities – the usual propaganda stunt – are promoting something called the WELL Health-Safety Rating to verify that a building or space has 'taken the necessary steps to prioritize the health and safety of their staff, visitors and other stakeholders'. They included Lady Gaga, Jennifer Lopez, Michael B. Jordan, Robert DeNiro, Venus Williams, Wolfgang Puck, Deepak Chopra and 17th Surgeon General Richard Carmona. Yawn. WELL Health-Safety has big connections with China. Parent company Delos is headed by former Goldman Sachs partner Paul Scialla. This is another example – and we will see so many others – of using the excuse of 'health' to dictate the lives and activities of the population. I guess one confirmation of the 'safety' of buildings is that only 'vaccinated' people can go in, right?

Electronic concentration camps

I wrote decades ago about the plans to restrict travel and here we are for those who refuse to bow to tyranny. This can be achieved in one go with air travel if the aviation industry makes a blanket decree.

The ‘vaccine’ and guaranteed income are designed to be part of a global version of China’s social credit system which tracks behaviour 24/7 and awards or deletes ‘credits’ based on whether your behaviour is supported by the state or not. I mean your entire lifestyle – what you do, eat, say, everything. Once your credit score falls below a certain level consequences kick in. In China tens of millions have been denied travel by air and train because of this. All the locations and activities denied to refusers by the ‘vaccine’ passports will be included in one big mass ban on doing almost anything for those that don’t bow their head to government. It’s beyond fascist and a new term is required to describe its extremes – I guess fascist technocracy will have to do. The way the Chinese system of technological – technocratic – control is sweeping the West can be seen in the Los Angeles school system and is planned to be expanded worldwide. Every child is required to have a ‘Covid’-tracking app scanned daily before they can enter the classroom. The so-called Daily Pass tracking system is produced by Gates’ Microsoft which I’m sure will shock you rigid. The pass will be scanned using a barcode (one step from an inside-the-body barcode) and the information will include health checks, ‘Covid’ tests and vaccinations. Entry codes are for one specific building only and access will only be allowed if a student or teacher has a negative test with a test not testing for the ‘virus’, has no symptoms of anything alleged to be related to ‘Covid’ (symptoms from a range of other illness), and has a temperature under 100 degrees. No barcode, no entry, is planned to be the case for everywhere and not only schools.

Kids are being psychologically prepared to accept this as ‘normal’ their whole life which is why what they can impose in schools is so important to the Cult and its gofers. Long-time American freedom campaigner John Whitehead of the Rutherford Institute was not exaggerating when he said: ‘Databit by databit, we are building our own electronic concentration camps.’ Canada under its Cult gofer prime minister Justin Trudeau has taken a major step towards the real thing with people interned against their will if they test positive with a test not testing for the ‘virus’ when they arrive at a Canadian

airport. They are jailed in internment hotels often without food or water for long periods and with many doors failing to lock there have been sexual assaults. The interned are being charged sometimes $2,000 for the privilege of being abused in this way. Trudeau is fully on board with the Cult and says the 'Covid pandemic' has provided an opportunity for a global 'reset' to permanently change Western civilisation. His number two, Deputy Prime Minister Chrystia Freeland, is a trustee of the World Economic Forum and a Rhodes Scholar. The Trudeau family have long been servants of the Cult. See *The Biggest Secret* and Cathy O'Brien's book *Trance-Formation of America* for the horrific background to Trudeau's father Pierre Trudeau another Canadian prime minister. Hide your fascism behind the façade of a heart-on-the-sleeve liberal. It's a well-honed Cult technique.

What can the 'vaccine' *really* do?

We have a 'virus' never shown to exist and 'variants' of the 'virus' that have also never been shown to exist except, like the 'original', as computer-generated fictions. Even if you believe there's a 'virus' the 'case' to 'death' rate is in the region of 0.23 to 0.15 percent and those 'deaths' are concentrated among the very old around the same average age that people die anyway. In response to this lack of threat (in truth none) psychopaths and idiots, knowingly and unknowingly answering to Gates and the Cult, are seeking to 'vaccinate' every man, woman and child on Planet Earth. Clearly the 'vaccine' is not about 'Covid' – none of this ever has been. So what is it all about *really*? Why the desperation to infuse genetically-manipulating synthetic material into everyone through mRNA fraudulent 'vaccines' with the intent of doing this over and over with the excuses of 'variants' and other 'virus' inventions? Dr Sherri Tenpenny, an osteopathic medical doctor in the United States, has made herself an expert on vaccines and their effects as a vehement campaigner against their use. Tenpenny was board certified in emergency medicine, the director of a level two trauma centre for 12 years, and moved to Cleveland in 1996 to start an integrative

medicine practice which has treated patients from all 50 states and some 17 other countries. Weaning people off pharmaceutical drugs is a speciality.

She became interested in the consequences of vaccines after attending a meeting at the National Vaccine Information Center in Washington DC in 2000 where she 'sat through four days of listening to medical doctors and scientists and lawyers and parents of vaccine injured kids' and asked: 'What's going on?' She had never been vaccinated and never got ill while her father was given a list of vaccines to be in the military and was 'sick his entire life'. The experience added to her questions and she began to examine vaccine documents from the Centers for Disease Control (CDC). After reading the first one, the 1998 version of *The General Recommendations of Vaccination*, she thought: 'This is it?' The document was poorly written and bad science and Tenpenny began 20 years of research into vaccines that continues to this day. She began her research into 'Covid vaccines' in March, 2020, and she describes them as 'deadly'. For many, as we have seen, they already have been. Tenpenny said that in the first 30 days of the 'vaccine' rollout in the United States there had been more than 40,000 adverse events reported to the vaccine adverse event database. A document had been delivered to her the day before that was 172 pages long. 'We have over 40,000 adverse events; we have over 3,100 cases of [potentially deadly] anaphylactic shock; we have over 5,000 neurological reactions.' Effects ranged from headaches to numbness, dizziness and vertigo, to losing feeling in hands or feet and paraesthesia which is when limbs 'fall asleep' and people have the sensation of insects crawling underneath their skin. All this happened in the first 30 days and remember that only about *ten percent* (or far less) of adverse reactions and vaccine-related deaths are estimated to be officially reported. Tenpenny said:

> So can you think of one single product in any industry, any industry, for as long as products have been made on the planet that within 30 days we have 40,000 people complaining of side effects that not only is still on the market but … we've got paid actors telling us how great

they are for getting their vaccine. We're offering people $500 if they will just get their vaccine and we've got nurses and doctors going; 'I got the vaccine, I got the vaccine'.

Tenpenny said they were not going to be 'happy dancing folks' when they began to suffer Bell's palsy (facial paralysis), neuropathies, cardiac arrhythmias and autoimmune reactions that kill through a blood disorder. 'They're not going to be so happy, happy then, but we're never going to see pictures of those people' she said. Tenpenny described the 'vaccine' as 'a well-designed killing tool'.

No off-switch

Bad as the initial consequences had been Tenpenny said it would be maybe 14 months before we began to see the 'full ravage' of what is going to happen to the 'Covid vaccinated' with full-out consequences taking anything between two years and 20 years to show. You can understand why when you consider that variations of the 'Covid vaccine' use mRNA (messenger RNA) to in theory activate the immune system to produce protective antibodies without using the actual 'virus'. How can they when it's a computer program and they've never isolated what they claim is the 'real thing'? Instead they use *synthetic* mRNA. They are inoculating synthetic material into the body which through a technique known as the Trojan horse is absorbed into cells to change the nature of DNA. Human DNA is changed by an infusion of messenger RNA and with each new 'vaccine' of this type it is changed even more. Say so and you are banned by Cult Internet platforms. The contempt the contemptuous Mark Zuckerberg has for the truth and human health can be seen in an internal Facebook video leaked to the Project Veritas investigative team in which he said of the 'Covid vaccines': '… I share some caution on this because we just don't know the long term side-effects of basically modifying people's DNA and RNA.' At the same time this disgusting man's Facebook was censoring and banning anyone saying exactly the same. He must go before a Nuremberg trial for crimes against humanity when he *knows* that he

is censoring legitimate concerns and denying the right of informed consent on behalf of the Cult that owns him. People have been killed and damaged by the very 'vaccination' technique he cast doubt on himself when they may not have had the 'vaccine' with access to information that he denied them. The plan is to have at least annual 'Covid vaccinations', add others to deal with invented 'variants', and change all other vaccines into the mRNA system. Pfizer executives told shareholders at a virtual Barclays Global Healthcare Conference in March, 2021, that the public may need a third dose of 'Covid vaccine', plus regular yearly boosters and the company planned to hike prices to milk the profits in a 'significant opportunity for our vaccine'. These are the professional liars, cheats and opportunists who are telling you their 'vaccine' is safe. Given this volume of mRNA planned to be infused into the human body and its ability to then replicate we will have a transformation of human genetics from biological to synthetic biological – exactly the long-time Cult plan for reasons we'll see – and many will die. Sherri Tenpenny said of this replication:

> It's like having an on-button but no off-button and that whole mechanism … they actually give it a name and they call it the Trojan horse mechanism, because it allows that [synthetic] virus and that piece of that [synthetic] virus to get inside of your cells, start to replicate and even get inserted into other parts of your DNA as a Trojan-horse.

Ask the overwhelming majority of people who have the 'vaccine' what they know about the contents and what they do and they would reply: 'The government says it will stop me getting the virus.' Governments give that false impression on purpose to increase take-up. You can read Sherri Tenpenny's detailed analysis of the health consequences in her blog at Vaxxter.com, but in summary these are some of them. She highlights the statement by Bill Gates about how human beings can become their own 'vaccine manufacturing machine'. The man is insane. ['Vaccine'-generated] 'antibodies' carry synthetic messenger RNA into the cells and the damage starts, Tenpenny contends, and she says that lungs can be adversely affected through varying degrees of pus and bleeding which

obviously affects breathing and would be dubbed 'Covid-19'. Even more sinister was the impact of 'antibodies' on macrophages, a white blood cell of the immune system. They consist of Type 1 and Type 2 which have very different functions. She said Type 1 are 'hyper-vigilant' white blood cells which 'gobble up' bacteria etc. However, in doing so, this could cause inflammation and in extreme circumstances be fatal. She says these affects are mitigated by Type 2 macrophages which kick in to calm down the system and stop it going rogue. They clear up dead tissue debris and reduce inflammation that the Type 1 'fire crews' have caused. Type 1 kills the infection and Type 2 heals the damage, she says. This is her punchline with regard to 'Covid vaccinations': She says that mRNA 'antibodies' block Type 2 macrophages by attaching to them and deactivating them. This meant that when the Type 1 response was triggered by infection there was nothing to stop that getting out of hand by calming everything down. There's an on-switch, but no off-switch, she says. What follows can be 'over and out, see you when I see you'.

Genetic suicide

Tenpenny also highlights the potential for autoimmune disease – the body attacking itself – which has been associated with vaccines since they first appeared. Infusing a synthetic foreign substance into cells could cause the immune system to react in a panic believing that the body is being overwhelmed by an invader (it is) and the consequences can again be fatal. There is an autoimmune response known as a 'cytokine storm' which I have likened to a homeowner panicked by an intruder and picking up a gun to shoot randomly in all directions before turning the fire on himself. The immune system unleashes a storm of inflammatory response called cytokines to a threat and the body commits hara-kiri. The lesson is that you mess with the body's immune response at your peril and these 'vaccines' seriously – fundamentally – mess with immune response. Tenpenny refers to a consequence called anaphylactic shock which is a severe and highly dangerous allergic reaction when the immune system

floods the body with chemicals. She gives the example of having a bee sting which primes the immune system and makes it sensitive to those chemicals. When people are stung again maybe years later the immune response can be so powerful that it leads to anaphylactic shock. Tenpenny relates this 'shock' with regard to the 'Covid vaccine' to something called polyethylene glycol or PEG. Enormous numbers of people have become sensitive to this over decades of use in a whole range of products and processes including food, drink, skin creams and 'medicine'. Studies have claimed that some 72 percent of people have antibodies triggered by PEG compared with two percent in the 1960s and allergic hypersensitive reactions to this become a gathering cause for concern. Tenpenny points out that the 'mRNA vaccine' is coated in a 'bubble' of polyethylene glycol which has the potential to cause anaphylactic shock through immune sensitivity. Many reports have appeared of people reacting this way after having the 'Covid vaccine'. What do we think is going to happen as humanity has more and more of these 'vaccines'? Tenpenny said: 'All these pictures we have seen with people with these rashes … these weepy rashes, big reactions on their arms and things like that – it's an acute allergic reaction most likely to the polyethylene glycol that you've been previously primed and sensitised to.'

Those who have not studied the conspiracy and its perpetrators at length might think that making the population sensitive to PEG and then putting it in these 'vaccines' is just a coincidence. It is not. It is instead testament to how carefully and coldly-planned current events have been and the scale of the conspiracy we are dealing with. Tenpenny further explains that the 'vaccine' mRNA procedure can breach the blood-brain barrier which protects the brain from toxins and other crap that will cause malfunction. In this case they could make two proteins corrupt brain function to cause Amyotrophic lateral sclerosis (ALS) , a progressive nervous system disease leading to loss of muscle control, and frontal lobe degeneration – Alzheimer's and dementia. Immunologist J. Bart Classon published a paper connecting mRNA 'vaccines' to prion

disease which can lead to Alzheimer's and other forms of neurogenerative disease while others have pointed out the potential to affect the placenta in ways that make women infertile. This will become highly significant in the next chapter when I will discuss other aspects of this non-vaccine that relate to its nanotechnology and transmission from the injected to the uninjected.

Qualified in idiocy

Tenpenny describes how research has confirmed that these 'vaccine'-generated antibodies can interact with a range of other tissues in the body and attack many other organs including the lungs. 'This means that if you have a hundred people standing in front of you that all got this shot they could have a hundred different symptoms.' Anyone really think that Cult gofers like the Queen, Tony Blair, Christopher Whitty, Anthony Fauci, and all the other psychopaths have really had this 'vaccine' in the pictures we've seen? Not a bloody chance. Why don't doctors all tell us about all these dangers and consequences of the 'Covid vaccine'? Why instead do they encourage and pressure patients to have the shot? Don't let's think for a moment that doctors and medical staff can't be stupid, lazy, and psychopathic and that's without the financial incentives to give the jab. Tenpenny again:

> Some people are going to die from the vaccine directly but a large number of people are going to start to get horribly sick and get all kinds of autoimmune diseases 42 days to maybe a year out. What are they going to do, these stupid doctors who say; 'Good for you for getting that vaccine.' What are they going to say; 'Oh, it must be a mutant, we need to give an extra dose of that vaccine.'
>
> Because now the vaccine, instead of one dose or two doses we need three or four because the stupid physicians aren't taking the time to learn anything about it. If I can learn this sitting in my living room reading a 19 page paper and several others so can they. There's nothing special about me, I just take the time to do it.

Remember how Sara Kayat, the NHS and TV doctor, said that the 'Covid vaccine' would '100 percent prevent hospitalisation and death'. Doctors can be idiots like every other profession and they

should not be worshipped as infallible. They are not and far from it. Behind many medical and scientific 'experts' lies an uninformed prat trying to hide themselves from you although in the 'Covid' era many have failed to do so as with UK narrative-repeating 'TV doctor' Hilary Jones. Pushing back against the minority of proper doctors and scientists speaking out against the 'vaccine' has been the entire edifice of the Cult global state in the form of governments, medical systems, corporations, mainstream media, Silicon Valley, and an army of compliant doctors, medical staff and scientists willing to say anything for money and to enhance their careers by promoting the party line. If you do that you are an 'expert' and if you won't you are an 'anti-vaxxer' and 'Covidiot'. The pressure to be 'vaccinated' is incessant. We have even had reports claiming that the 'vaccine' can help cure cancer and Alzheimer's and make the lame walk. I am waiting for the announcement that it can bring you coffee in the morning and cook your tea. Just as the symptoms of 'Covid' seem to increase by the week so have the miracles of the 'vaccine'. American supermarket giant Kroger Co. offered nearly 500,000 employees in 35 states a $100 bonus for having the 'vaccine' while donut chain Krispy Kreme promised 'vaccinated' customers a free glazed donut every day for the rest of 2021. Have your DNA changed and you will get a doughnut although we might not have to give you them for long. Such offers and incentives confirm the desperation.

Perhaps the worse vaccine-stunt of them all was UK 'Health' Secretary Matt-the-prat Hancock on live TV after watching a clip of someone being 'vaccinated' when the roll-out began. Hancock faked tears so badly it was embarrassing. Brain-of-Britain Piers Morgan, the lockdown-supporting, 'vaccine' supporting, 'vaccine' passport-supporting, TV host played along with Hancock – 'You're quite emotional about that' he said in response to acting so atrocious it would have been called out at a school nativity which will presumably today include Mary and Jesus in masks, wise men keeping their camels six feet apart, and shepherds under tent arrest. System-serving Morgan tweeted this: 'Love the idea of covid vaccine passports for everywhere: flights, restaurants, clubs, football, gyms,

shops etc. It's time covid-denying, anti-vaxxer loonies had their bullsh*t bluff called & bar themselves from going anywhere that responsible citizens go.' If only I could aspire to his genius. To think that Morgan, who specialises in shouting over anyone he disagrees with, was lauded as a free speech hero when he lost his job after storming off the set of his live show like a child throwing his dolly out of the pram. If he is a free speech hero we are in real trouble. I have no idea what 'bullsh*t' means, by the way, the * throws me completely.

The Cult is desperate to infuse its synthetic DNA-changing concoction into everyone and has been using every lie, trick and intimidation to do so. The question of '*Why*?' we shall now address.

CHAPTER TEN

Human 2.0

I believe that at the end of the century the use of words and general educated opinion will have altered so much that one will be able to speak of machines thinking without expecting to be contradicted –
Alan Turing (1912-1954), the 'Father of artificial intelligence'

I have been exposing for decades the plan to transform the human body from a biological to a synthetic-biological state. The new human that I will call Human 2.0 is planned to be connected to artificial intelligence and a global AI 'Smart Grid' that would operate as one global system in which AI would control everything from your fridge to your heating system to your car to your mind. Humans would no longer be 'human', but post-human and sub-human, with their thinking and emotional processes replaced by AI.

What I said sounded crazy and beyond science fiction and I could understand that. To any balanced, rational, mind it *is* crazy. Today, however, that world is becoming reality and it puts the 'Covid vaccine' into its true context. Ray Kurzweil is the ultra-Zionist 'computer scientist, inventor and futurist' and co-founder of the Singularity University. Singularity refers to the merging of humans with machines or 'transhumanism'. Kurzweil has said humanity would be connected to the cyber 'cloud' in the period of the ever-recurring year of 2030:

Our thinking … will be a hybrid of biological and non-biological thinking … humans will be able to extend their limitations and 'think in the cloud' … We're going to put gateways to the

cloud in our brains ... We're going to gradually merge and enhance ourselves ... In my view, that's the nature of being human – we transcend our limitations. As the technology becomes vastly superior to what we are then the small proportion that is still human gets smaller and smaller and smaller until it's just utterly negligible.

They are trying to sell this end-of-humanity-as-we-know-it as the next stage of 'evolution' when we become super-human and 'like the gods'. They are lying to you. Shocked, eh? The population, and again especially the young, have been manipulated into addiction to technologies designed to enslave them for life. First they induced an addiction to smartphones (holdables); next they moved to technology on the body (wearables); and then began the invasion of the body (implantables). I warned way back about the plan for microchipped people and we are now entering that era. We should not be diverted into thinking that this refers only to chips we can see. Most important are the nanochips known as smart dust, neural dust and nanobots which are far too small to be seen by the human eye. Nanotechnology is everywhere, increasingly in food products, and released into the atmosphere by the geoengineering of the skies funded by Bill Gates to 'shut out the Sun' and 'save the planet from global warming'. Gates has been funding a project to spray millions of tonnes of chalk (calcium carbonate) into the stratosphere over Sweden to 'dim the Sun' and cool the Earth. Scientists warned the move could be disastrous for weather systems in ways no one can predict and opposition led to the Swedish space agency announcing that the 'experiment' would not be happening as planned in the summer of 2021; but it shows where the Cult is going with dimming the impact of the Sun and there's an associated plan to change the planet's atmosphere. Who gives psychopath Gates the right to dictate to the entire human race and dismantle planetary systems? The world will not be safe while this man is at large.

The global warming hoax has made the Sun, like the gas of life, something to fear when both are essential to good health and human survival (more inversion). The body transforms sunlight into vital vitamin D through a process involving … *cholesterol*. This is the cholesterol we are also told to fear. We are urged to take Big Pharma

statin drugs to reduce cholesterol and it's all systematic. Reducing cholesterol means reducing vitamin D uptake with all the multiple health problems that will cause. At least if you take statins long term it saves the government from having to pay you a pension. The delivery system to block sunlight is widely referred to as chemtrails although these have a much deeper agenda, too. They appear at first to be contrails or condensation trails streaming from aircraft into cold air at high altitudes. Contrails disperse very quickly while chemtrails do not and spread out across the sky before eventually their content falls to earth. Many times I have watched aircraft cross-cross a clear blue sky releasing chemtrails until it looks like a cloudy day. Chemtrails contain many things harmful to humans and the natural world including toxic heavy metals, aluminium (see Alzheimer's) and nanotechnology. Ray Kurzweil reveals the reason without actually saying so: 'Nanobots will infuse all the matter around us with information. Rocks, trees, everything will become these intelligent creatures.' How do you deliver that? *From the sky*. Self-replicating nanobots would connect everything to the Smart Grid. The phenomenon of Morgellons disease began in the chemtrail era and the correlation has led to it being dubbed the 'chemtrail disease'. Self-replicating fibres appear in the body that can be pulled out through the skin. Morgellons fibres continue to grow outside the body and have a form of artificial intelligence. I cover this at greater length in *Phantom Self*.

'Vaccine' operating system

'Covid vaccines' with their self-replicating synthetic material are also designed to make the connection between humanity and Kurzweil's 'cloud'. American doctor and dedicated campaigner for truth, Carrie Madej, an Internal Medicine Specialist in Georgia with more than 20 years medical experience, has highlighted the nanotechnology aspect of the fake 'vaccines'. She explains how one of the components in at least the Moderna and Pfizer synthetic potions are 'lipid nanoparticles' which are 'like little tiny computer bits' – a 'sci-fi substance' known as nanobots and hydrogel which can be 'triggered

at any moment to deliver its payload' and act as 'biosensors'. The synthetic substance had 'the ability to accumulate data from your body like your breathing, your respiration, thoughts and emotions, all kind of things' and each syringe could carry a *million* nanobots:

This substance because it's like little bits of computers in your body, crazy, but it's true, it can do that, [and] obviously has the ability to act through Wi-Fi. It can receive and transmit energy, messages, frequencies or impulses. That issue has never been addressed by these companies. What does that do to the human?

Just imagine getting this substance in you and it can react to things all around you, the 5G, your smart device, your phones, what is happening with that? What if something is triggering it, too, like an impulse, a frequency? We have something completely foreign in the human body.

Madej said her research revealed that electromagnetic (EMF) frequencies emitted by phones and other devices had increased dramatically in the same period of the 'vaccine' rollout and she was seeing more people with radiation problems as 5G and other electromagnetic technology was expanded and introduced to schools and hospitals. She said she was 'floored with the EMF coming off' the devices she checked. All this makes total sense and syncs with my own work of decades when you think that Moderna refers in documents to its mRNA 'vaccine' as an 'operating system':

Recognizing the broad potential of mRNA science, we set out to create an mRNA technology platform that functions very much like an operating system on a computer. It is designed so that it can plug and play interchangeably with different programs. In our case, the 'program' or 'app' is our mRNA drug – the unique mRNA sequence that codes for a protein …

… Our MRNA Medicines – 'The 'Software Of Life': When we have a concept for a new mRNA medicine and begin research, fundamental components are already in place. Generally, the only thing that changes from one potential mRNA medicine to another is the coding region – the actual genetic code that instructs ribosomes to make protein. Utilizing these instruction sets gives our investigational mRNA medicines a software-like quality. We also have the ability to combine different mRNA sequences encoding for different proteins in a single mRNA investigational medicine.

Who needs a real 'virus' when you can create a computer version to justify infusing your operating system into the entire human race on the road to making living, breathing people into cyborgs? What is missed with the 'vaccines' is the *digital* connection between synthetic material and the body that I highlighted earlier with the study that hacked a computer with human DNA. On one level the body is digital, based on mathematical codes, and I'll have more about that in the next chapter. Those who ridiculously claim that mRNA 'vaccines' are not designed to change human genetics should explain the words of Dr Tal Zaks, chief medical officer at Moderna, in a 2017 TED talk. He said that over the last 30 years 'we've been living this phenomenal digital scientific revolution, and I'm here today to tell you, that we are actually *hacking the software of life*, and that it's changing the way we think about prevention and treatment of disease':

In every cell there's this thing called messenger RNA, or mRNA for short, that transmits the critical information from the DNA in our genes to the protein, which is really the stuff we're all made out of. This is the critical information that determines what the cell will do. So we think about it as an operating system. So if you could change that, if you could introduce a line of code, or change a line of code, it turns out, that has profound implications for everything, from the flu to cancer.

Zaks should more accurately have said that this has profound implications for the human genetic code and the nature of DNA. Communications within the body go both ways and not only one. But, hey, no, the 'Covid vaccine' will not affect your genetics. Cult fact-checkers say so even though the man who helped to develop the mRNA technique says that it does. Zaks said in 2017:

If you think about what it is we're trying to do. We've taken information and our understanding of that information and how that information is transmitted in a cell, and we've taken our understanding of medicine and how to make drugs, and we're fusing the two. We think of it as information therapy.

I have been writing for decades that the body is an information field communicating with itself and the wider world. This is why

radiation which is information can change the information field of body and mind through phenomena like 5G and change their nature and function. 'Information therapy' means to change the body's information field and change the way it operates. DNA is a receiver-transmitter of information and can be mutated by information like mRNA synthetic messaging. Technology to do this has been ready and waiting in the underground bases and other secret projects to be rolled out when the 'Covid' hoax was played. 'Trials' of such short and irrelevant duration were only for public consumption. When they say the 'vaccine' is 'experimental' that is not true. It may appear to be 'experimental' to those who don't know what's going on, but the trials have already been done to ensure the Cult gets the result it desires. Zaks said that it took decades to sequence the human genome, completed in 2003, but now they could do it in a week. By 'they' he means scientists operating in the public domain. In the secret projects they were sequencing the genome in a week long before even 2003.

Deluge of mRNA

Highly significantly the Moderna document says the guiding premise is that if using mRNA as a medicine works for one disease then it should work for many diseases. They were leveraging the flexibility afforded by their platform and the fundamental role mRNA plays in protein synthesis to pursue mRNA medicines for a broad spectrum of diseases. Moderna is confirming what I was saying through 2020 that multiple 'vaccines' were planned for 'Covid' (and later invented 'variants') and that previous vaccines would be converted to the mRNA system to infuse the body with massive amounts of genetically-manipulating synthetic material to secure a transformation to a synthetic-biological state. The 'vaccines' are designed to kill stunning numbers as part of the long-exposed Cult depopulation agenda and transform the rest. Given this is the goal you can appreciate why there is such hysterical demand for every human to be 'vaccinated' for an alleged 'disease' that has an estimated 'infection' to 'death' ratio of 0.23-0.15 percent. As I write

children are being given the 'vaccine' in trials (their parents are a disgrace) and ever-younger people are being offered the vaccine for a 'virus' that even if you believe it exists has virtually zero chance of harming them. Horrific effects of the 'trials' on a 12-year-old girl were revealed by a family member to be serious brain and gastric problems that included a bowel obstruction and the inability to swallow liquids or solids. She was unable to eat or drink without throwing up, had extreme pain in her back, neck and abdomen, and was paralysed from the waist down which stopped her urinating unaided. When the girl was first taken to hospital doctors said it was all in her mind. She was signed up for the 'trial' by her parents for whom no words suffice. None of this 'Covid vaccine' insanity makes any sense unless you see what the 'vaccine' really is – a body-changer. Synthetic biology or 'SynBio' is a fast-emerging and expanding scientific discipline which includes everything from genetic and molecular engineering to electrical and computer engineering. Synthetic biology is defined in these ways:

- A multidisciplinary area of research that seeks to create new biological parts, devices, and systems, or to redesign systems that are already found in nature.

- The use of a mixture of physical engineering and genetic engineering to create new (and therefore synthetic) life forms.

- An emerging field of research that aims to combine the knowledge and methods of biology, engineering and related disciplines in the design of chemically-synthesized DNA to create organisms with novel or enhanced characteristics and traits (synthetic organisms including humans).

We now have synthetic blood, skin, organs and limbs being developed along with synthetic body parts produced by 3D printers. These are all elements of the synthetic human programme and this comment by Kurzweil's co-founder of the Singularity University,

Peter Diamandis, can be seen in a whole new light with the 'Covid' hoax and the sanctions against those that refuse the 'vaccine':

Anybody who is going to be resisting the progress forward [to transhumanism] is going to be resisting evolution and, fundamentally, they will die out. It's not a matter of whether it's good or bad. It's going to happen.

'Resisting evolution'? What absolute bollocks. The arrogance of these people is without limit. His 'it's going to happen' mantra is another way of saying 'resistance is futile' to break the spirit of those pushing back and we must not fall for it. Getting this genetically-transforming 'vaccine' into everyone is crucial to the Cult plan for total control and the desperation to achieve that is clear for anyone to see. Vaccine passports are a major factor in this and they, too, are a form of resistance is futile. It's NOT. The paper funded by the Rockefeller Foundation for the 2013 'health conference' in China said:

We will interact more with artificial intelligence. The use of robotics, bio-engineering to augment human functioning is already well underway and will advance. Re-engineering of humans into potentially separate and unequal forms through genetic engineering or mixed human-robots raises debates on ethics and equality.

A new demography is projected to emerge after 2030 [that year again] of technologies (robotics, genetic engineering, nanotechnology) producing robots, engineered organisms, 'nanobots' and artificial intelligence (AI) that can self-replicate. Debates will grow on the implications of an impending reality of human designed life.

What is happening today is so long planned. The world army enforcing the will of the world government is intended to be a robot army, not a human one. Today's military and its technologically 'enhanced' troops, pilotless planes and driverless vehicles are just stepping stones to that end. Human soldiers are used as Cult fodder and its time they woke up to that and worked for the freedom of the population instead of their own destruction and their family's destruction – the same with the police. Join us and let's sort this out. The phenomenon of enforce my own destruction is widespread in the 'Covid' era with Woker 'luvvies' in the acting and entertainment

industries supporting 'Covid' rules which have destroyed their profession and the same with those among the public who put signs on the doors of their businesses 'closed due to Covid – stay safe' when many will never reopen. It's a form of masochism and most certainly insanity.

Transgender = transhumanism

When something explodes out of nowhere and is suddenly everywhere it is always the Cult agenda and so it is with the tidal wave of claims and demands that have infiltrated every aspect of society under the heading of 'transgenderism'. The term 'trans' is so 'in' and this is the dictionary definition:

A prefix meaning 'across', 'through', occurring … in loanwords from Latin, used in particular for denoting movement or conveyance from place to place (transfer; transmit; transplant) or complete change (transform; transmute), or to form adjectives meaning 'crossing', 'on the other side of', or 'going beyond' the place named (transmontane; transnational; trans-Siberian).

Transgender means to go beyond gender and transhuman means to go beyond human. Both are aspects of the Cult plan to transform the human body to a synthetic state with *no gender*. Human 2.0 is not designed to procreate and would be produced technologically with no need for parents. The new human would mean the end of parents and so men, and increasingly women, are being targeted for the deletion of their rights and status. Parental rights are disappearing at an ever-quickening speed for the same reason. The new human would have no need for men or women when there is no procreation and no gender. Perhaps the transgender movement that appears to be in a permanent state of frenzy might now contemplate on how it is being used. This was never about transgender rights which are only the interim excuse for confusing gender, particularly in the young, on the road to *fusing* gender. Transgender activism is not an end; it is a *means* to an end. We see again the technique of creative destruction in which you destroy the status quo to 'build back better' in the form that you want. The gender status quo had to be

destroyed by persuading the Cult-created Woke mentality to believe that you can have 100 genders or more. A programme for 9 to 12 year olds produced by the Cult-owned BBC promoted the 100 genders narrative. The very idea may be the most monumental nonsense, but it is not what is true that counts, only what you can make people *believe* is true. Once the gender of 2 + 2 = 4 has been dismantled through indoctrination, intimidation and 2 + 2 = 5 then the new no-gender normal can take its place with Human 2.0. Aldous Huxley revealed the plan in his prophetic *Brave New World* in 1932:

> Natural reproduction has been done away with and children are created, decanted', and raised in 'hatcheries and conditioning centres'. From birth, people are genetically designed to fit into one of five castes, which are further split into 'Plus' and 'Minus' members and designed to fulfil predetermined positions within the social and economic strata of the World State.

How could Huxley know this in 1932? For the same reason George Orwell knew about the Big Brother state in 1948, Cult insiders I have quoted knew about it in 1969, and I have known about it since the early 1990s. If you are connected to the Cult or you work your balls off to uncover the plan you can predict the future. The process is simple. If there is a plan for the world and nothing intervenes to stop it then it will happen. Thus if you communicate the plan ahead of time you are perceived to have predicted the future, but you haven't. You have revealed the plan which without intervention will become the human future. The whole reason I have done what I have is to alert enough people to inspire an intervention and maybe at last that time has come with the Cult and its intentions now so obvious to anyone with a brain in working order.

The future is here

Technological wombs that Huxley described to replace parent procreation are already being developed and they are only the projects we know about in the public arena. Israeli scientists told *The Times of Israel* in March, 2021, that they have grown 250-cell embryos

into mouse foetuses with fully formed organs using artificial wombs in a development they say could pave the way for gestating humans outside the womb. Professor Jacob Hanna of the Weizmann Institute of Science said:

We took mouse embryos from the mother at day five of development, when they are just of 250 cells, and had them in the incubator from day five until day 11, by which point they had grown all their organs.

By day 11 they make their own blood and have a beating heart, a fully developed brain. Anybody would look at them and say, 'this is clearly a mouse foetus with all the characteristics of a mouse.' It's gone from being a ball of cells to being an advanced foetus.

A special liquid is used to nourish embryo cells in a laboratory dish and they float on the liquid to duplicate the first stage of embryonic development. The incubator creates all the right conditions for its development, Hanna said. The liquid gives the embryo 'all the nutrients, hormones and sugars they need' along with a custom-made electronic incubator which controls gas concentration, pressure and temperature. The cutting-edge in the underground bases and other secret locations will be light years ahead of that, however, and this was reported by the London *Guardian* in 2017:

We are approaching a biotechnological breakthrough. Ectogenesis, the invention of a complete external womb, could completely change the nature of human reproduction. In April this year, researchers at the Children's Hospital of Philadelphia announced their development of an artificial womb.

The article was headed 'Artificial wombs could soon be a reality. What will this mean for women?' What would it mean for children is an even bigger question. No mother to bond with only a machine in preparation for a life of soulless interaction and control in a world governed by machines (see the *Matrix* movies). Now observe the calculated manipulations of the 'Covid' hoax as human interaction and warmth has been curtailed by distancing, isolation and fear with people communicating via machines on a scale never seen before.

These are all dots in the same picture as are all the personal assistants, gadgets and children's toys through which kids and adults communicate with AI as if it is human. The AI 'voice' on Sat-Nav should be included. All these things are psychological preparation for the Cult endgame. Before you can make a physical connection with AI you have to make a psychological connection and that is what people are being conditioned to do with this ever gathering human-AI interaction. Movies and TV programmes depicting the transhuman, robot dystopia relate to a phenomenon known as 'pre-emptive programming' in which the world that is planned is portrayed everywhere in movies, TV and advertising. This is conditioning the conscious and subconscious mind to become familiar with the planned reality to dilute resistance when it happens for real. What would have been a shock such is the change is made less so. We have young children put on the road to transgender transition surgery with puberty blocking drugs at an age when they could never be able to make those life-changing decisions.

Rachel Levine, a professor of paediatrics and psychiatry who believes in treating children this way, became America's highest-ranked openly-transgender official when she was confirmed as US Assistant Secretary at the Department of Health and Human Services after being nominated by Joe Biden (the Cult). Activists and governments press for laws to deny parents a say in their children's transition process so the kids can be isolated and manipulated into agreeing to irreversible medical procedures. A Canadian father Robert Hoogland was denied bail by the Vancouver Supreme Court in 2021 and remained in jail for breaching a court order that he stay silent over his young teenage daughter, a minor, who was being offered life-changing hormone therapy without parental consent. At the age of 12 the girl's 'school counsellor' said she may be transgender, referred her to a doctor and told the school to treat her like a boy. This is another example of state-serving schools imposing ever more control over children's lives while parents have ever less.

Contemptible and extreme child abuse is happening all over the world as the Cult gender-fusion operation goes into warp-speed.

Why the war on men – and now women?

The question about what artificial wombs mean for women should rightly be asked. The answer can be seen in the deletion of women's rights involving sport, changing rooms, toilets and status in favour of people in male bodies claiming to identify as women. I can identify as a mountain climber, but it doesn't mean I can climb a mountain any more than a biological man can be a biological woman. To believe so is a triumph of belief over factual reality which is the very perceptual basis of everything Woke. Women's sport is being destroyed by allowing those with male bodies who say they identify as female to 'compete' with girls and women. Male body 'women' dominate 'women's' competition with their greater muscle mass, bone density, strength and speed. With that disadvantage sport for women loses all meaning. To put this in perspective nearly 300 American high school boys can run faster than the quickest woman sprinter in the world. Women are seeing their previously protected spaces invaded by male bodies simply because they claim to identify as women. That's all they need to do to access all women's spaces and activities under the Biden 'Equality Act' that destroys equality for women with the usual Orwellian Woke inversion. Male sex offenders have already committed rapes in women's prisons after claiming to identify as women to get them transferred. Does this not matter to the Woke 'equality' hypocrites? Not in the least. What matters to Cult manipulators and funders behind transgender activists is to advance gender fusion on the way to the no-gender 'human'. When you are seeking to impose transparent nonsense like this, or the 'Covid' hoax, the only way the nonsense can prevail is through censorship and intimidation of dissenters, deletion of factual information, and programming of the unquestioning, bewildered and naive. You don't have to scan the world for long to see that all these things are happening.

Many women's rights organisations have realised that rights and status which took such a long time to secure are being eroded and that it is systematic. Kara Dansky of the global Women's Human Rights Campaign said that Biden's transgender executive order immediately he took office, subsequent orders, and Equality Act legislation that followed 'seek to erase women and girls in the law as a category'. *Exactly*. I said during the long ago-started war on men (in which many women play a crucial part) that this was going to turn into a war on them. The Cult is phasing out *both* male and female genders. To get away with that they are brought into conflict so they are busy fighting each other while the Cult completes the job with no unity of response. Unity, people, *unity*. We need unity everywhere. Transgender is the only show in town as the big step towards the no-gender human. It's not about rights for transgender people and never has been. Woke political correctness is deleting words relating to genders to the same end. Wokers believe this is to be 'inclusive' when the opposite is true. They are deleting words describing gender because gender *itself* is being deleted by Human 2.0. Terms like 'man', 'woman', 'mother' and 'father' are being deleted in the universities and other institutions to be replaced by the *no*-gender, not trans-gender, 'individuals' and 'guardians'. Women's rights campaigner Maria Keffler of Partners for Ethical Care said: 'Children are being taught from kindergarten upward that some boys have a vagina, some girls have a penis, and that kids can be any gender they want to be.' Do we really believe that suddenly countries all over the world at the same time had the idea of having drag queens go into schools or read transgender stories to very young children in the local library? It's coldly-calculated confusion of gender on the way to the fusion of gender. Suzanne Vierling, a psychologist from Southern California, made another important point:

Yesterday's slave woman who endured gynecological medical experiments is today's girl-child being butchered in a booming gender-transitioning sector. Ovaries removed, pushing her into menopause and osteoporosis, uncharted territory, and parents' rights and authority decimated.

The erosion of parental rights is a common theme in line with the Cult plans to erase the very concept of parents and 'ovaries removed, pushing her into menopause' means what? Those born female lose the ability to have children – another way to discontinue humanity as we know it.

Eliminating Human 1.0 (before our very eyes)

To pave the way for Human 2.0 you must phase out Human 1.0. This is happening through plummeting sperm counts and making women infertile through an onslaught of chemicals, radiation (including smartphones in pockets of men) and mRNA 'vaccines'. Common agriculture pesticides are also having a devastating impact on human fertility. I have been tracking collapsing sperm counts in the books for a long time and in 2021 came a book by fertility scientist and reproductive epidemiologist Shanna Swan, *Count Down: How Our Modern World Is Threatening Sperm Counts, Altering Male and Female Reproductive Development and Imperiling the Future of the Human Race*. She reports how the global fertility rate dropped by *half* between 1960 and 2016 with America's birth rate 16 percent below where it needs to be to sustain the population. Women are experiencing declining egg quality, more miscarriages, and more couples suffer from infertility. Other findings were an increase in erectile dysfunction, infant boys developing more genital abnormalities, male problems with conception, and plunging levels of the male hormone testosterone which would explain why so many men have lost their backbone and masculinity. This has been very evident during the 'Covid' hoax when women have been prominent among the Pushbackers and big strapping blokes have bowed their heads, covered their faces with a nappy and quietly submitted. Mind control expert Cathy O'Brien also points to how global education introduced the concept of 'we're all winners' in sport and classrooms: 'Competition was defused, and it in turn defused a sense of fighting back.' This is another version of the 'equity' doctrine in which you drive down rather than raise up. What a contrast in Cult-controlled China with its global ambitions

where the government published plans in January, 2021, to 'cultivate masculinity' in boys from kindergarten through to high school in the face of a 'masculinity crisis'. A government adviser said boys would be soon become 'delicate, timid and effeminate' unless action was taken. Don't expect any similar policy in the targeted West. A 2006 study showed that a 65-year-old man in 2002 had testosterone levels *15 percent* lower than a 65-year-old man in 1987 while a 2020 study found a similar story with young adults and adolescents. Men are getting prescriptions for testosterone replacement therapy which causes an even greater drop in sperm count with up to 99 percent seeing sperm counts drop to zero during the treatment. More sperm is defective and malfunctioning with some having two heads or not pursuing an egg.

A class of *synthetic* chemicals known as phthalates are being blamed for the decline. These are found everywhere in plastics, shampoos, cosmetics, furniture, flame retardants, personal care products, pesticides, canned foods and even receipts. Why till receipts? Everyone touches them. Let no one delude themselves that all this is not systematic to advance the long-time agenda for human body transformation. Phthalates mimic hormones and disrupt the hormone balance causing testosterone to fall and genital birth defects in male infants. Animals and fish have been affected in the same way due to phthalates and other toxins in rivers. When fish turn gay or change sex through chemicals in rivers and streams it is a pointer to why there has been such an increase in gay people and the sexually confused. It doesn't matter to me what sexuality people choose to be, but if it's being affected by chemical pollution and consumption then we need to know. Does anyone really think that this is not connected to the transgender agenda, the war on men and the condemnation of male 'toxic masculinity'? You watch this being followed by 'toxic femininity'. It's already happening. When breastfeeding becomes 'chest-feeding', pregnant women become pregnant people along with all the other Woke claptrap you know that the world is going insane and there's a Cult scam in progress. Transgender activists are promoting the Cult agenda while Cult

billionaires support and fund the insanity as they laugh themselves to sleep at the sheer stupidity for which humans must be infamous in galaxies far, far away.

'Covid vaccines' and female infertility

We can now see why the 'vaccine' has been connected to potential infertility in women. Dr Michael Yeadon, former Vice President and Chief Scientific Advisor at Pfizer, and Dr Wolfgang Wodarg in Germany, filed a petition with the European Medicines Agency in December, 2020, urging them to stop trials for the Pfizer/BioNTech shot and all other mRNA trials until further studies had been done. They were particularly concerned about possible effects on fertility with 'vaccine'-produced antibodies attacking the protein Syncytin-1 which is responsible for developing the placenta. The result would be infertility 'of indefinite duration' in women who have the 'vaccine' with the placenta failing to form. Section 10.4.2 of the Pfizer/BioNTech trial protocol says that pregnant women or those who might become so should not have mRNA shots. Section 10.4 warns men taking mRNA shots to 'be abstinent from heterosexual intercourse' and not to donate sperm. The UK government said that it *did not know* if the mRNA procedure had an effect on fertility. *Did not know?* These people have to go to jail. UK government advice did not recommend at the start that pregnant women had the shot and said they should avoid pregnancy for at least two months after 'vaccination'. The 'advice' was later updated to pregnant women should only have the 'vaccine' if the benefits outweighed the risks to mother and foetus. What the hell is that supposed to mean? Then 'spontaneous abortions' began to appear and rapidly increase on the adverse reaction reporting schemes which include only a fraction of adverse reactions. Thousands and ever-growing numbers of 'vaccinated' women are describing changes to their menstrual cycle with heavier blood flow, irregular periods and menstruating again after going through the menopause – all links to reproduction effects. Women are passing blood clots and the lining of their uterus while men report erectile dysfunction and blood effects. Most

significantly of all *un*vaccinated women began to report similar menstrual changes after interaction with *'vaccinated'* people and men and children were also affected with bleeding noses, blood clots and other conditions. 'Shedding' is when vaccinated people can emit the content of a vaccine to affect the unvaccinated, but this is different. 'Vaccinated' people were not shedding a 'live virus' allegedly in 'vaccines' as before because the fake 'Covid vaccines' involve synthetic material and other toxicity. Doctors exposing what is happening prefer the term 'transmission' to shedding. Somehow those that have had the shots are transmitting effects to those that haven't. Dr Carrie Madej said the nano-content of the 'vaccines' can 'act like an antenna' to others around them which fits perfectly with my own conclusions. This 'vaccine' transmission phenomenon was becoming known as the book went into production and I deal with this further in the Postscript.

Vaccine effects on sterility are well known. The World Health Organization was accused in 2014 of sterilising millions of women in Kenya with the evidence confirmed by the content of the vaccines involved. The same WHO behind the 'Covid' hoax admitted its involvement for more than ten years with the vaccine programme. Other countries made similar claims. Charges were lodged by Tanzania, Nicaragua, Mexico, and the Philippines. The Gardasil vaccine claimed to protect against a genital 'virus' known as HPV has also been linked to infertility. Big Pharma and the WHO (same thing) are criminal and satanic entities. Then there's the Bill Gates Foundation which is connected through funding and shared interests with 20 pharmaceutical giants and laboratories. He stands accused of directing the policy of United Nations Children's Fund (UNICEF), vaccine alliance GAVI, and other groupings, to advance the vaccine agenda and silence opposition at great cost to women and children. At the same time Gates wants to reduce the global population. Coincidence?

Great Reset = Smart Grid = new human

The Cult agenda I have been exposing for 30 years is now being openly promoted by Cult assets like Gates and Klaus Schwab of the World Economic Forum under code-terms like the 'Great Reset', 'Build Back Better' and 'a rare but narrow window of opportunity to reflect, reimagine, and reset our world'. What provided this 'rare but narrow window of opportunity'? The 'Covid' hoax did. Who created that? *They* did. My books from not that long ago warned about the planned 'Internet of Things' (IoT) and its implications for human freedom. This was the plan to connect all technology to the Internet and artificial intelligence and today we are way down that road with an estimated 36 billion devices connected to the World Wide Web and that figure is projected to be 76 billion by 2025. I further warned that the Cult planned to go beyond that to the Internet of *Everything* when the human brain was connected via AI to the Internet and Kurzweil's 'cloud'. Now we have Cult operatives like Schwab calling for precisely that under the term 'Internet of Bodies', a fusion of the physical, digital and biological into one centrally-controlled Smart Grid system which the Cult refers to as the 'Fourth Industrial Revolution'. They talk about the 'biological', but they really mean the synthetic-biological which is required to fully integrate the human body and brain into the Smart Grid and artificial intelligence planned to replace the human mind. We have everything being synthetically manipulated including the natural world through GMO and smart dust, the food we eat and the human body itself with synthetic 'vaccines'. I said in *The Answer* that we would see the Cult push for synthetic meat to replace animals and in February, 2021, the so predictable psychopath Bill Gates called for the introduction of synthetic meat to save us all from 'climate change'. The climate hoax just keeps on giving like the 'Covid' hoax. The war on meat by vegan activists is a carbon (oops, sorry) copy of the manipulation of transgender activists. They have no idea (except their inner core) that they are being used to promote and impose the agenda of the Cult or that they are only the *vehicle* and not the *reason*. This is not to say those who choose not to eat meat shouldn't be respected and supported in that right, but there are ulterior motives

for those in power. A *Forbes* article in December, 2019, highlighted the plan so beloved of Schwab and the Cult under the heading: 'What Is The Internet of Bodies? And How Is It Changing Our World?' The article said the human body is the latest data platform (remember 'our vaccine is an operating system'). *Forbes* described the plan very accurately and the words could have come straight out of my books from long before:

The Internet of Bodies (IoB) is an extension of the IoT and basically connects the human body to a network through devices that are ingested, implanted, or connected to the body in some way. Once connected, data can be exchanged, and the body and device can be remotely monitored and controlled.

They were really describing a human hive mind with human perception centrally-dictated via an AI connection as well as allowing people to be 'remotely monitored and controlled'. Everything from a fridge to a human mind could be directed from a central point by these insane psychopaths and 'Covid vaccines' are crucial to this. Forbes explained the process I mentioned earlier of holdable and wearable technology followed by implantable. The article said there were three generations of the Internet of Bodies that include:

- Body external: These are wearable devices such as Apple Watches or Fitbits that can monitor our health.

- Body internal: These include pacemakers, cochlear implants, and digital pills that go inside our bodies to monitor or control various aspects of health.

- Body embedded: The third generation of the Internet of Bodies is embedded technology where technology and the human body are melded together and have a real-time connection to a remote machine.

Forbes noted the development of the Brain Computer Interface (BCI) which merges the brain with an external device for monitoring and controlling in real-time. 'The ultimate goal is to help restore function to individuals with disabilities by using brain signals rather than conventional neuromuscular pathways.' Oh, do fuck off. The goal of brain interface technology is controlling human thought and emotion from the central point in a hive mind serving its masters wishes. Many people are now agreeing to be chipped to open doors without a key. You can recognise them because they'll be wearing a mask, social distancing and lining up for the 'vaccine'. The Cult plans a Great Reset money system after they have completed the demolition of the global economy in which 'money' will be exchanged through communication with body operating systems. Rand Corporation, a Cult-owned think tank, said of the Internet of Bodies or IoB:

> Internet of Bodies technologies fall under the broader IoT umbrella. But as the name suggests, IoB devices introduce an even more intimate interplay between humans and gadgets. IoB devices monitor the human body, collect health metrics and other personal information, and transmit those data over the Internet. Many devices, such as fitness trackers, are already in use … IoB devices … and those in development can track, record, and store users' whereabouts, bodily functions, and what they see, hear, and even think.

Schwab's World Economic Forum, a long-winded way of saying 'fascism' or 'the Cult', has gone full-on with the Internet of Bodies in the 'Covid' era. 'We're entering the era of the Internet of Bodies', it declared, 'collecting our physical data via a range of devices that can be implanted, swallowed or worn'. The result would be a huge amount of health-related data that could improve human wellbeing around the world, and prove crucial in fighting the 'Covid-19 pandemic'. Does anyone think these clowns care about 'human wellbeing' after the death and devastation their pandemic hoax has purposely caused? Schwab and co say we should move forward with the Internet of Bodies because 'Keeping track of symptoms could help us stop the spread of infection, and quickly detect new cases'. How wonderful, but keeping track' is all they are really bothered

about. Researchers were investigating if data gathered from smartwatches and similar devices could be used as viral infection alerts by tracking the user's heart rate and breathing. Schwab said in his 2018 book *Shaping the Future of the Fourth Industrial Revolution*:

> The lines between technologies and beings are becoming blurred and not just by the ability to create lifelike robots or synthetics. Instead it is about the ability of new technologies to literally become part of us. Technologies already influence how we understand ourselves, how we think about each other, and how we determine our realities. As the technologies … give us deeper access to parts of ourselves, we may begin to integrate digital technologies into our bodies.

You can see what the game is. Twenty-four hour control and people – if you could still call them that – would never know when something would go ping and take them out of circulation. It's the most obvious rush to a global fascist dictatorship and the complete submission of humanity and yet still so many are locked away in their Cult-induced perceptual coma and can't see it.

Smart Grid control centres

The human body is being transformed by the 'vaccines' and in other ways into a synthetic cyborg that can be attached to the global Smart Grid which would be controlled from a central point and other sub-locations of Grid manipulation. Where are these planned to be? Well, China for a start which is one of the Cult's biggest centres of operation. The technological control system and technocratic rule was incubated here to be unleashed across the world after the 'Covid' hoax came out of China in 2020. Another Smart Grid location that will surprise people new to this is Israel. I have exposed in *The Trigger* how Sabbatian technocrats, intelligence and military operatives were behind the horrors of 9/11 and not '19 Arab hijackers' who somehow manifested the ability to pilot big passenger airliners when instructors at puddle-jumping flying schools described some of them as a joke. The 9/11 attacks were made possible through control of civilian and military air computer systems and those of the White House, Pentagon and connected agencies. See *The Trigger* – it

will blow your mind. The controlling and coordinating force were the Sabbatian networks in Israel and the United States which by then had infiltrated the entire US government, military and intelligence system. The real name of the American Deep State is 'Sabbatian State'. Israel is a tiny country of only nine million people, but it is one of the global centres of cyber operations and fast catching Silicon Valley in importance to the Cult. Israel is known as the 'start-up nation' for all the cyber companies spawned there with the Sabbatian specialisation of 'cyber security' that I mentioned earlier which gives those companies access to computer systems of their clients in real time through 'backdoors' written into the coding when security software is downloaded. The Sabbatian centre of cyber operations outside Silicon Valley is the Israeli military Cyber Intelligence Unit, the biggest infrastructure project in Israel's history, headquartered in the desert-city of Beersheba and involving some 20,000 'cyber soldiers'. Here are located a literal army of Internet trolls scanning social media, forums and comment lists for anyone challenging the Cult agenda. The UK military has something similar with its 77th Brigade and associated operations. The Beersheba complex includes research and development centres for other Cult operations such as Intel, Microsoft, IBM, Google, Apple, Hewlett-Packard, Cisco Systems, Facebook and Motorola. Techcrunch.com ran an article about the Beersheba global Internet technology centre headlined 'Israel's desert city of Beersheba is turning into a cybertech oasis':

> The military's massive relocation of its prestigious technology units, the presence of multinational and local companies, a close proximity to Ben Gurion University and generous government subsidies are turning Beersheba into a major global cybertech hub. Beersheba has all of the ingredients of a vibrant security technology ecosystem, including Ben Gurion University with its graduate program in cybersecurity and Cyber Security Research Center, and the presence of companies such as EMC, Deutsche Telekom, PayPal, Oracle, IBM, and Lockheed Martin. It's also the future home of the INCB (Israeli National Cyber Bureau); offers a special income tax incentive for cyber security companies, and was the site for the relocation of the army's intelligence corps units.

Sabbatians have taken over the cyber world through the following process: They scan the schools for likely cyber talent and develop them at Ben Gurion University and their period of conscription in the Israeli Defense Forces when they are stationed at the Beersheba complex. When the cyber talented officially leave the army they are funded to start cyber companies with technology developed by themselves or given to them by the state. Much of this is stolen through backdoors of computer systems around the world with America top of the list. Others are sent off to Silicon Valley to start companies or join the major ones and so we have many major positions filled by apparently 'Jewish' but really Sabbatian operatives. Google, YouTube and Facebook are all run by 'Jewish' CEOs while Twitter is all but run by ultra-Zionist hedge-fund shark Paul Singer. At the centre of the Sabbatian global cyber web is the Israeli army's Unit 8200 which specialises in hacking into computer systems of other countries, inserting viruses, gathering information, instigating malfunction, and even taking control of them from a distance. A long list of Sabbatians involved with 9/11, Silicon Valley and Israeli cyber security companies are operatives of Unit 8200. This is not about Israel. It's about the Cult. Israel is planned to be a Smart Grid hub as with China and what is happening at Beersheba is not for the benefit of Jewish people who are treated disgustingly by the Sabbatian elite that control the country. A glance at the Nuremberg Codes will tell you that.

The story is much bigger than 'Covid', important as that is to where we are being taken. Now, though, it's time to really strap in. There's more … much more …

CHAPTER ELEVEN

Who controls the Cult?

Awake, arise or be forever fall'n
John Milton, *Paradise Lost*

I have exposed this far the level of the Cult conspiracy that operates in the world of the seen and within the global secret society and satanic network which operates in the shadows one step back from the seen. The story, however, goes much deeper than that.

The 'Covid' hoax is major part of the Cult agenda, but only part, and to grasp the biggest picture we have to expand our attention beyond the realm of human sight and into the infinity of possibility that we cannot see. It is from here, ultimately, that humanity is being manipulated into a state of total control by the force which dictates the actions of the Cult. How much of reality can we see? Next to damn all is the answer. We may appear to see all there is to see in the 'space' our eyes survey and observe, but little could be further from the truth. The human 'world' is only a tiny band of frequency that the body's visual and perceptual systems can decode into *perception* of a 'world'. According to mainstream science the electromagnetic spectrum is 0.005 percent of what exists in the Universe (Fig 10). The maximum estimate I have seen is 0.5 percent and either way it's miniscule. I say it is far, far, smaller even than 0.005 percent when you compare reality we see with the totality of reality that we don't. Now get this if you are new to such information: Visible light, the only band of frequency that we can see, is a *fraction* of the 0.005

percent (Fig 11 overleaf). Take this further and realise that our universe is one of infinite universes and that universes are only a fragment of overall reality – *infinite* reality. Then compare that with the almost infinitesimal frequency band of visible light or human sight. You see that humans are as near blind as it is possible to be without actually being so. Artist and filmmaker, Sergio Toporek, said:

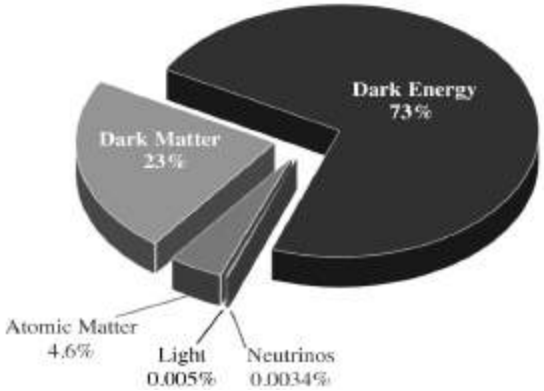


Figure 10: Humans can perceive such a tiny band of visual reality it's laughable.

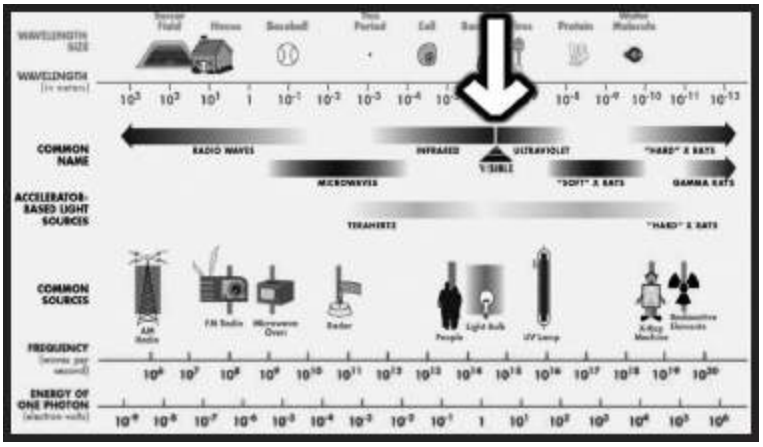


Figure 11: We can see a smear of the 0.005 percent electromagnetic spectrum, but we still know it all. Yep, makes sense.

Consider that you can see less than 1% of the electromagnetic spectrum and hear less than 1% of the acoustic spectrum. 90% of the cells in your body carry their own microbial DNA and are not 'you'. The atoms in your body are 99.9999999999999999% empty space and none of them are the ones you were born with ... Human beings have 46 chromosomes, two less than a potato.

The existence of the rainbow depends on the conical photoreceptors in your eyes; to animals without cones, the rainbow does not exist. So you don't just look at a rainbow, you create it. This is pretty amazing, especially considering that all the beautiful colours you see represent less than 1% of the electromagnetic spectrum.

Suddenly the 'world' of humans looks a very different place. Take into account, too, that Planet Earth when compared with the projected size of this single universe is the equivalent of a billionth of a pinhead. Imagine the ratio that would be when compared to infinite reality. To think that Christianity once insisted that Earth and humanity were the centre of everything. This background is vital if we are going to appreciate the nature of 'human' and how we can be manipulated by an unseen force. To human visual reality virtually *everything* is unseen and yet the prevailing perception within the institutions and so much of the public is that if we can't see it, touch it, hear it, taste it and smell it then it cannot exist. Such perception is indoctrinated and encouraged by the Cult and its agents because it isolates believers in the strictly limited, village-idiot, realm of the five senses where perceptions can be firewalled and information controlled. Most of those perpetuating the 'this-world-is-all-there-is' insanity are themselves indoctrinated into believing the same delusion. While major players and influencers know that official reality is laughable most of those in science, academia and medicine really believe the nonsense they peddle and teach succeeding generations. Those who challenge the orthodoxy are dismissed as nutters and freaks to protect the manufactured illusion from exposure. Observe the dynamic of the 'Covid' hoax and you will see how that takes the same form. The inner-circle psychopaths knows it's a gigantic scam, but almost the entirety of those imposing their fascist rules believe that 'Covid' is all that they're told it is.

Stolen identity

Ask people who they are and they will give you their name, place of birth, location, job, family background and life story. Yet that is not who they are – it is what they are *experiencing*. The difference is *absolutely crucial*. The true 'I', the eternal, infinite 'I', is consciousness,

a state of being aware. Forget 'form'. That is a vehicle for a brief experience. Consciousness does not come *from* the brain, but *through* the brain and even that is more symbolic than literal. We are awareness, pure awareness, and this is what withdraws from the body at what we call 'death' to continue our eternal beingness, *isness*, in other realms of reality within the limitlessness of infinity or the Biblical 'many mansions in my father's house'. Labels of a human life, man, woman, transgender, black, white, brown, nationality, circumstances and income are not who we are. They are what we are – awareness – is *experiencing* in a brief connection with a band of frequency we call 'human'. The labels are not the self; they are, to use the title of one of my books, a *Phantom* Self. I am not David Icke born in Leicester, England, on April 29th, 1952. I am the consciousness *having that experience*. The Cult and its non-human masters seek to convince us through the institutions of 'education', science, medicine, media and government that what we are *experiencing* is who we *are*. It's so easy to control and direct perception locked away in the bewildered illusions of the five senses with no expanded radar. Try, by contrast, doing the same with a humanity aware of its true self and its true power to consciously create its reality and experience. How is it possible to do this? We do it all day every day. If you perceive yourself as 'little me' with no power to impact upon your life and the world then your life experience will reflect that. You will hand the power you don't think you have to authority in all its forms which will use it to control your experience. This, in turn, will appear to confirm your perception of 'little me' in a self-fulfilling feedback loop. But that is what 'little me' really is – a *perception*. We are all 'big-me', infinite me, and the Cult has to make us forget that if its will is to prevail. We are therefore manipulated and pressured into self-identifying with human labels and not the consciousness/awareness *experiencing* those human labels.

The phenomenon of identity politics is a Cult-instigated manipulation technique to sub-divide previous labels into even smaller ones. A United States university employs this list of letters to

describe student identity: LGBTTQQFAGPBDSM or lesbian, gay, bisexual, transgender, transsexual, queer, questioning, flexual, asexual, gender-fuck, polyamorous, bondage/discipline, dominance/submission and sadism/masochism. I'm sure other lists are even longer by now as people feel the need to self-identity the 'I' with the minutiae of race and sexual preference. Wokers programmed by the Cult for generations believe this is about 'inclusivity' when it's really the Cult locking them away into smaller and smaller versions of Phantom Self while firewalling them from the influence of their true self, the infinite, eternal 'I'. You may notice that my philosophy which contends that we are all unique points of attention/awareness within the same infinite whole or Oneness is the ultimate non-racism. The very sense of Oneness makes the judgement of people by their body-type, colour or sexuality utterly ridiculous and confirms that racism has no understanding of reality (including anti-white racism). Yet despite my perception of life Cult agents and fast-asleep Wokers label me racist to discredit my information while they are themselves phenomenally racist and sexist. All they see is race and sexuality and they judge people as good or bad, demons or untouchables, by their race and sexuality. All they see is *Phantom Self* and perceive themselves in terms of Phantom Self. They are pawns and puppets of the Cult agenda to focus attention and self-identity in the five senses and play those identities against each other to divide and rule. Columbia University has introduced segregated graduations in another version of social distancing designed to drive people apart and teach them that different racial and cultural groups have nothing in common with each other. The last thing the Cult wants is unity. Again the pump-primers of this will be Cult operatives in the knowledge of what they are doing, but the rest are just the Phantom Self blind leading the Phantom Self blind. We *do* have something in common – we are all *the same consciousness* having different temporary experiences.

What is this 'human'?

Yes, what *is* 'human'? That is what we are supposed to be, right? I mean 'human'? True, but 'human' is the experience not the 'I'. Break it down to basics and 'human' is the way that information is processed. If we are to experience and interact with this band of frequency we call the 'world' we must have a vehicle that operates within that band of frequency. Our consciousness in its prime form cannot do that; it is way beyond the frequency of the human realm. My consciousness or awareness could not tap these keys and pick up the cup in front of me in the same way that radio station A cannot interact with radio station B when they are on different frequencies. The human body is the means through which we have that interaction. I have long described the body as a biological computer which processes information in a way that allows consciousness to experience this reality. The body is a receiver, transmitter and processor of information in a particular way that we call human. We visually perceive only the world of the five senses in a wakened state – that is the limit of the body's visual decoding system. In truth it's not even visual in the way we experience 'visual reality' as I will come to in a moment. We are 'human' because the body processes the information sources of human into a reality and behaviour system that we *perceive* as human. Why does an elephant act like an elephant and not like a human or a duck? The elephant's biological computer is a different information field and processes information according to that program into a visual and behaviour type we call an elephant. The same applies to everything in our reality. These body information fields are perpetuated through procreation (like making a copy of a software program). The Cult wants to break that cycle and intervene technologically to transform the human information field into one that will change what we call humanity. If it can change the human information field it will change the way that field processes information and change humanity both 'physically' and psychologically. Hence the *messenger* (information) RNA 'vaccines' and so much more that is targeting human genetics by changing the body's information – *messaging* – construct through food, drink, radiation, toxicity and other means.

Reality that we experience is nothing like reality as it really is in the same way that the reality people experience in virtual reality games is not the reality they are really living in. The game is only a decoded source of information that appears to be a reality. Our world is also an information construct – a *simulation* (more later). In its base form our reality is a wavefield of information much the same in theme as Wi-Fi. The five senses decode wavefield information into electrical information which they communicate to the brain to decode into holographic (illusory 'physical') information. Different parts of the brain specialise in decoding different senses and the information is fused into a reality that appears to be outside of us but is really inside the brain and the genetic structure in general (Fig 12 overleaf). DNA is a receiver-transmitter of information and a vital part of this decoding process and the body's connection to other realities. Change DNA and you change the way we decode and connect with reality – see 'Covid vaccines'. Think of computers decoding Wi-Fi. You have information encoded in a radiation field and the computer decodes that information into a very different form on the screen. You can't see the Wi-Fi until its information is made manifest on the screen and the information on the screen is inside the computer and not outside. I have just described how we decode the 'human world'. All five senses decode the waveform 'Wi-Fi' field into electrical signals and the brain (computer) constructs reality inside the brain and not outside – 'You don't just look at a rainbow, you create it'. Sound is a simple example. We don't hear sound until the brain decodes it. Waveform sound waves are picked up by the hearing sense and communicated to the brain in an electrical form to be decoded into the sounds that we hear. Everything we hear is inside the brain along with everything we see, feel, smell and taste. Words and language are waveform fields generated by our vocal chords which pass through this process until they are decoded by the brain into words that we hear. Different languages are different frequency fields or sound waves generated by vocal chords. Late British philosopher Alan Watts said:

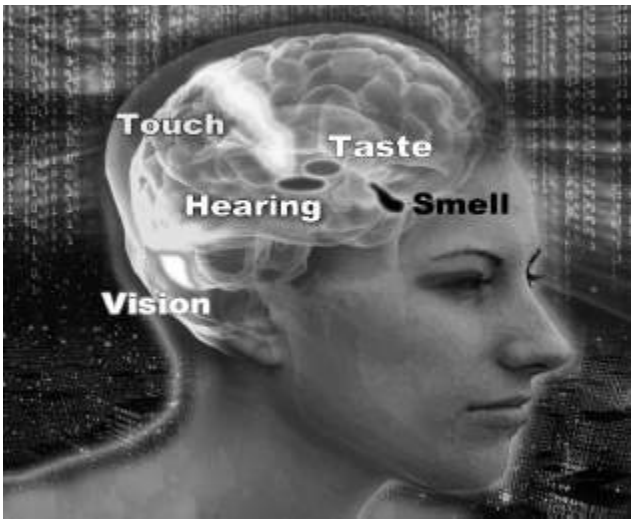


Figure 12: The brain receives information from the five senses and constructs from that our perceived reality.

[Without the brain] the world is devoid of light, heat, weight, solidity, motion, space, time or any other imaginable feature. All these phenomena are interactions, or transactions, of vibrations with a certain arrangement of neurons.

That's exactly what they are and scientist Robert Lanza describes in his book, *Biocentrism*, how we decode electromagnetic waves and energy into visual and 'physical' experience. He uses the example of a flame emitting photons, electromagnetic energy, each pulsing electrically and magnetically:

… these … invisible electromagnetic waves strike a human retina, and if (and only if) the waves happen to measure between 400 and 700 nano meters in length from crest to crest, then their energy is just right to deliver a stimulus to the 8 million cone-shaped cells in the retina.

Each in turn send an electrical pulse to a neighbour neuron, and on up the line this goes, at 250 mph, until it reaches the … occipital lobe of the brain, in the back of the head. There, a cascading complex of neurons fire from the incoming stimuli, and we subjectively perceive this experience as a yellow brightness occurring in a place we have been conditioned to call the 'external world'.

You hear what you decode

If a tree falls or a building collapses they make no noise unless someone is there to decode the energetic waves generated by the disturbance into what we call sound. Does a falling tree make a noise? Only if you hear it – *decode* it. Everything in our reality is a frequency field of information operating within the overall 'Wi-Fi' field that I call The Field. A vibrational disturbance is generated in The Field by the fields of the falling tree or building. These disturbance waves are what we decode into the sound of them falling. If no one is there to do that then neither will make any noise. Reality is created by the observer – *decoder* – and the *perceptions* of the observer affect the decoding process. For this reason different people – different *perceptions* – will perceive the same reality or situation in a different way. What one may perceive as a nightmare another will see as an opportunity. The question of why the Cult is so focused on controlling human perception now answers itself. All experienced reality is the act of decoding and we don't experience Wi-Fi until it is decoded on the computer screen. The sight and sound of an Internet video is encoded in the Wi-Fi all around us, but we don't see or hear it until the computer decodes that information. Taste, smell and touch are all phenomena of the brain as a result of the same process. We don't taste, smell or feel anything except in the brain and there are pain relief techniques that seek to block the signal from the site of discomfort to the brain because if the brain doesn't decode that signal we don't feel pain. Pain is in the brain and only appears to be at the point of impact thanks to the feedback loop between them. We don't see anything until electrical information from the sight senses is decoded in an area at the back of the brain. If that area is damaged we can go blind when our eyes are perfectly okay. So why do we go blind if we damage an eye? We damage the information processing between the waveform visual information and the visual decoding area of the brain. If information doesn't reach the brain in a form it can decode then we can't see the visual reality that it represents. What's more the brain is decoding only a fraction of the information it receives and the rest is absorbed by the

sub-conscious mind. This explanation is from the science magazine, *Wonderpedia*:

> Every second, 11 million sensations crackle along these [brain] pathways ... The brain is confronted with an alarming array of images, sounds and smells which it rigorously filters down until it is left with a manageable list of around 40. Thus 40 sensations per second make up what we perceive as reality.

The 'world' is not what people are told to believe that is it and the inner circles of the Cult *know that*.

Illusory 'physical' reality

We can only see a smear of 0.005 percent of the Universe which is only one of a vast array of universes – 'mansions' – within infinite reality. Even then the brain decodes only 40 pieces of information ('sensations') from a potential *11 million* that we receive every second. Two points strike you from this immediately: The sheer breathtaking stupidity of believing we know anything so rigidly that there's nothing more to know; and the potential for these processes to be manipulated by a malevolent force to control the reality of the population. One thing I can say for sure with no risk of contradiction is that when you can perceive an almost indescribable fraction of infinite reality there is always more to know as in tidal waves of it. Ancient Greek philosopher Socrates was so right when he said that wisdom is to know how little we know. How obviously true that is when you think that we are experiencing a physical world of solidity that is neither physical nor solid and a world of apartness when everything is connected. Cult-controlled 'science' dismisses the so-called 'paranormal' and all phenomena related to that when the 'para'-normal is perfectly normal and explains the alleged 'great mysteries' which dumbfound scientific minds. There is a reason for this. A 'scientific mind' in terms of the mainstream is a material mind, a five-sense mind imprisoned in see it, touch it, hear it, smell it and taste it. Phenomena and happenings that can't be explained that way leave the 'scientific mind' bewildered and the rule is that if they

can't account for why something is happening then it can't, by definition, be happening. I beg to differ. Telepathy is thought waves passing through The Field (think wave disturbance again) to be decoded by someone able to connect with that wavelength (information). For example: You can pick up the thought waves of a friend at any distance and at the very least that will bring them to mind. A few minutes later the friend calls you. 'My god', you say, 'that's incredible – I was just thinking of you.' Ah, but *they* were thinking of *you* before they made the call and that's what you decoded. Native peoples not entrapped in five-sense reality do this so well it became known as the 'bush telegraph'. Those known as psychics and mediums (genuine ones) are doing the same only across dimensions of reality. 'Mind over matter' comes from the fact that matter and mind are the *same*. The state of one influences the state of the other. Indeed one *and* the other are illusions. They are aspects of the same field. Paranormal phenomena are all explainable so why are they still considered 'mysteries' or not happening? Once you go down this road of understanding you begin to expand awareness beyond the five senses and that's the nightmare for the Cult.

Figure 13: Holograms are not solid, but the best ones appear to be.

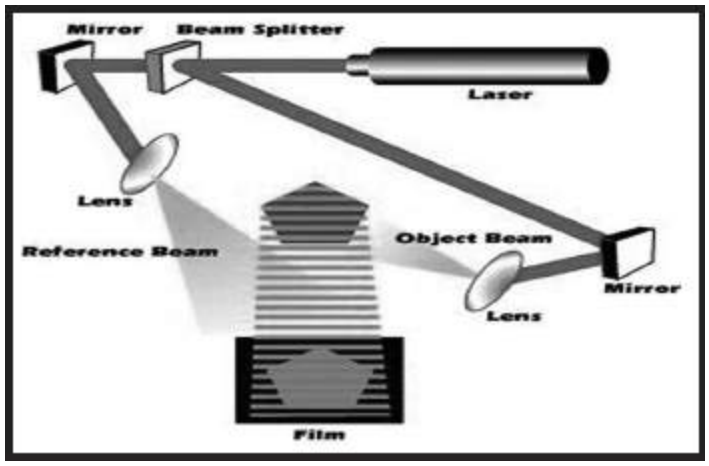


Figure 14: How holograms are created by capturing a waveform version of the subject image.

Holographic 'solidity'

Our reality is not solid, it is holographic. We are now well aware of holograms which are widely used today. Two-dimensional information is decoded into a three-dimensional reality that is not solid although can very much appear to be (Fig 13). Holograms are created with a laser divided into two parts. One goes directly onto a holographic photographic print ('reference beam') and the other takes a waveform image of the subject ('working beam') before being directed onto the print where it 'collides' with the other half of the laser (Fig 14). This creates a *waveform* interference pattern which contains the wavefield information of whatever is being photographed (Fig 15 overleaf). The process can be likened to dropping pebbles in a pond. Waves generated by each one spread out across the water to collide with the others and create a wave representation of where the stones fell and at what speed, weight and distance. A waveform interference pattern of a hologram is akin to the waveform information in The Field which the five senses decode into electrical signals to be decoded by the brain into a holographic illusory 'physical' reality. In the same way when a laser (think human attention) is directed at the waveform interference pattern a three-dimensional version of the subject is projected into apparently 'solid' reality (Fig 16). An amazing trait of holograms reveals more 'paranormal mysteries'. Information of the *whole*

hologram is encoded in waveform in every part of the interference pattern by the way they are created. This means that every *part* of a hologram is a smaller version of the whole. Cut the interference wave-pattern into four and you won't get four parts of the image. You get quarter-sized versions of the *whole* image. The body is a hologram and the same applies. Here we have the basis of acupuncture, reflexology and other forms of healing which identify representations of the whole body in all of the parts, hands, feet, ears, everywhere. Skilled palm readers can do what they do because the information of whole body is encoded in the hand. The concept of as above, so below, comes from this.

Figure 15: A waveform interference pattern that holds the information that transforms into a hologram.

Figure 16: Holographic people including 'Elvis' holographically inserted to sing a duet with Celine Dion.

The question will be asked of why, if solidity is illusory, we can't just walk through walls and each other. The resistance is not solid against solid; it is electromagnetic field against electromagnetic field and we decode this into the *experience* of solid against solid. We should also not underestimate the power of belief to dictate reality. What you believe is impossible *will be*. Your belief impacts on your decoding processes and they won't decode what you think is impossible. What we believe we perceive and what we perceive we experience. 'Can't dos' and 'impossibles' are like a firewall in a computer system that won't put on the screen what the firewall blocks. How vital that is to understanding how human experience has been hijacked. I explain in *The Answer*, *Everything You Need To Know But Have Never Been Told* and other books a long list of 'mysteries' and 'paranormal' phenomena that are not mysterious and perfectly normal once you realise what reality is and how it works. 'Ghosts' can be seen to pass through 'solid' walls because the walls are not solid and the ghost is a discarnate entity operating on a frequency so different to that of the wall that it's like two radio stations sharing the same space while never interfering with each other. I have seen ghosts do this myself. The apartness of people and objects is also an illusion. Everything is connected by the Field like all sea life is connected by the sea. It's just that within the limits of our visual reality we only 'see' holographic information and not the field of information that connects everything and from which the holographic world is made manifest. If you can only see holographic 'objects' and not the field that connects them they will appear to you as unconnected to each other in the same way that we see the computer while not seeing the Wi-Fi.

What you don't know *can* hurt you

Okay, we return to those 'two worlds' of human society and the Cult with its global network of interconnecting secret societies and satanic groups which manipulate through governments, corporations, media, religions, etc. The fundamental difference between them is *knowledge*. The idea has been to keep humanity

ignorant of the plan for its total enslavement underpinned by a crucial ignorance of reality – who we are and where we are – and how we interact with it. 'Human' should be the interaction between our expanded eternal consciousness and the five-sense body experience. We are meant to be *in* this world in terms of the five senses but not *of* this world in relation to our greater consciousness and perspective. In that state we experience the small picture of the five senses within the wider context of the big picture of awareness beyond the five senses. Put another way the five senses see the dots and expanded awareness connects them into pictures and patterns that give context to the apparently random and unconnected. Without the context of expanded awareness the five senses see only apartness and randomness with apparently no meaning. The Cult and its other-dimensional controllers seek to intervene in the frequency realm where five-sense reality is supposed to connect with expanded reality and to keep the two apart (more on this in the final chapter). When that happens five-sense mental and emotional processes are no longer influenced by expanded awareness, or the True 'I', and instead are driven by the isolated perceptions of the body's decoding systems. They are in the world *and* of it. Here we have the human plight and why humanity with its potential for infinite awareness can be so easily manipulatable and descend into such extremes of stupidity.

Once the Cult isolates five-sense mind from expanded awareness it can then program the mind with perceptions and beliefs by controlling information that the mind receives through the 'education' system of the formative years and the media perceptual bombardment and censorship of an entire lifetime. Limit perception and a sense of the possible through limiting knowledge by limiting and skewing information while censoring and discrediting that which could set people free. As the title of another of my books says *… And The Truth Shall Set You Free*. For this reason the last thing the Cult wants in circulation is the truth about anything – especially the reality of the eternal 'I' – and that's why it is desperate to control information. The Cult knows that information becomes perception

which becomes behaviour which, collectively, becomes human society. Cult-controlled and funded mainstream 'science' denies the existence of an eternal 'I' and seeks to dismiss and trash all evidence to the contrary. Cult-controlled mainstream religion has a version of 'God' that is little more than a system of control and dictatorship that employs threats of damnation in an afterlife to control perceptions and behaviour in the here and now through fear and guilt. Neither is true and it's the 'neither' that the Cult wishes to suppress. This 'neither' is that everything is an expression, a point of attention, within an infinite state of consciousness which is the real meaning of the term 'God'.

Perceptual obsession with the 'physical body' and five-senses means that 'God' becomes personified as a bearded bloke sitting among the clouds or a raging bully who loves us if we do what 'he' wants and condemns us to the fires of hell if we don't. These are no more than a 'spiritual' fairy tales to control and dictate events and behaviour through fear of this 'God' which has bizarrely made 'God-fearing' in religious circles a state to be desired. I would suggest that fearing *anything* is not to be encouraged and celebrated, but rather deleted. You can see why 'God fearing' is so beneficial to the Cult and its religions when *they* decide what 'God' wants and what 'God' demands (the Cult demands) that everyone do. As the great American comedian Bill Hicks said satirising a Christian zealot: 'I think what God meant to say.' How much of this infinite awareness ('God') that we access is decided by how far we choose to expand our perceptions, self-identity and sense of the possible. The scale of self-identity reflects itself in the scale of awareness that we can connect with and are influenced by – how much knowing and insight we have instead of programmed perception. You cannot expand your awareness into the infinity of possibility when you believe that you are little me Peter the postman or Mary in marketing and nothing more. I'll deal with this in the concluding chapter because it's crucial to how we turnaround current events.

Where the Cult came from

When I realised in the early 1990s there was a Cult network behind global events I asked the obvious question: When did it start? I took it back to ancient Rome and Egypt and on to Babylon and Sumer in Mesopotamia, the 'Land Between Two Rivers', in what we now call Iraq. The two rivers are the Tigris and Euphrates and this region is of immense historical and other importance to the Cult, as is the land called Israel only 550 miles away by air. There is much more going with deep esoteric meaning across this whole region. It's not only about 'wars for oil'. Priceless artefacts from Mesopotamia were stolen or destroyed after the American and British invasion of Iraq in 2003 justified by the lies of Boy Bush and Tony Blair (their Cult masters) about non-existent 'weapons of mass destruction'. Mesopotamia was the location of Sumer (about 5,400BC to 1,750BC), and Babylon (about 2,350BC to 539BC). Sabbatians may have become immensely influential in the Cult in modern times but they are part of a network that goes back into the mists of history. Sumer is said by historians to be the 'cradle of civilisation'. I disagree. I say it was the re-start of what we call human civilisation after cataclysmic events symbolised in part as the 'Great Flood' destroyed the world that existed before. These fantastic upheavals that I have been describing in detail in the books since the early1990s appear in accounts and legends of ancient cultures across the world and they are supported by geological and biological evidence. Stone tablets found in Iraq detailing the Sumer period say the cataclysms were caused by non-human 'gods' they call the Anunnaki. These are described in terms of extraterrestrial visitations in which knowledge supplied by the Anunnaki is said to have been the source of at least one of the world's oldest writing systems and developments in astronomy, mathematics and architecture that were way ahead of their time. I have covered this subject at length in *The Biggest Secret* and *Children of the Matrix* and the same basic 'Anunnaki' story can be found in Zulu accounts in South Africa where the late and very great Zulu high shaman Credo Mutwa told me that the Sumerian Anunnaki were known by Zulus as the Chitauri or 'children of the serpent'. See my six-hour video interview with Credo on this subject entitled *The*

Reptilian Agenda recorded at his then home near Johannesburg in 1999 which you can watch on the Ickonic media platform.

The Cult emerged out of Sumer, Babylon and Egypt (and elsewhere) and established the Roman Empire before expanding with the Romans into northern Europe from where many empires were savagely imposed in the form of Cult-controlled societies all over the world. Mass death and destruction was their calling card. The Cult established its centre of operations in Europe and European Empires were Cult empires which allowed it to expand into a global force. Spanish and Portuguese colonialists headed for Central and South America while the British and French targeted North America. Africa was colonised by Britain, France, Belgium, the Netherlands, Portugal, Spain, Italy, and Germany. Some like Britain and France moved in on the Middle East. The British Empire was by far the biggest for a simple reason. By now Britain was the headquarters of the Cult from which it expanded to form Canada, the United States, Australia and New Zealand. The Sun never set on the British Empire such was the scale of its occupation. London remains a global centre for the Cult along with Rome and the Vatican although others have emerged in Israel and China. It is no accident that the 'virus' is alleged to have come out of China while Italy was chosen as the means to terrify the Western population into compliance with 'Covid' fascism. Nor that Israel has led the world in 'Covid' fascism and mass 'vaccination'.

You would think that I would mention the United States here, but while it has been an important means of imposing the Cult's will it is less significant than would appear and is currently in the process of having what power it does have deleted. The Cult in Europe has mostly loaded the guns for the US to fire. America has been controlled from Europe from the start through Cult operatives in Britain and Europe. The American Revolution was an illusion to make it appear that America was governing itself while very different forces were pulling the strings in the form of Cult families such as the Rothschilds through the Rockefellers and other subordinates. The Rockefellers are extremely close to Bill Gates and

established both scalpel and drug 'medicine' and the World Health Organization. They play a major role in the development and circulation of vaccines through the Rockefeller Foundation on which Bill Gates said his Foundation is based. Why wouldn't this be the case when the Rockefellers and Gates are on the same team? Cult infiltration of human society goes way back into what we call history and has been constantly expanding and centralising power with the goal of establishing a global structure to dictate everything. Look how this has been advanced in great leaps with the 'Covid' hoax.

The non-human dimension

I researched and observed the comings and goings of Cult operatives through the centuries and even thousands of years as they were born, worked to promote the agenda within the secret society and satanic networks, and then died for others to replace them. Clearly there had to be a coordinating force that spanned this entire period while operatives who would not have seen the end goal in their lifetimes came and went advancing the plan over millennia. I went in search of that coordinating force with the usual support from the extraordinary synchronicity of my life which has been an almost daily experience since 1990. I saw common themes in religious texts and ancient cultures about a non-human force manipulating human society from the hidden. Christianity calls this force Satan, the Devil and demons; Islam refers to the Jinn or Djinn; Zulus have their Chitauri (spelt in other ways in different parts of Africa); and the Gnostic people in Egypt in the period around and before 400AD referred to this phenomena as the 'Archons', a word meaning rulers in Greek. Central American cultures speak of the 'Predators' among other names and the same theme is everywhere. I will use 'Archons' as a collective name for all of them. When you see how their nature and behaviour is described all these different sources are clearly talking about the same force. Gnostics described the Archons in terms of 'luminous fire' while Islam relates the Jinn to 'smokeless fire'. Some refer to beings in form that could occasionally be seen, but the most common of common theme is that they operate from

unseen realms which means almost all existence to the visual processes of humans. I had concluded that this was indeed the foundation of human control and that the Cult was operating within the human frequency band on behalf of this hidden force when I came across the writings of Gnostics which supported my conclusions in the most extraordinary way.

A sealed earthen jar was found in 1945 near the town of Nag Hammadi about 75-80 miles north of Luxor on the banks of the River Nile in Egypt. Inside was a treasure trove of manuscripts and texts left by the Gnostic people some 1,600 years earlier. They included 13 leather-bound papyrus codices (manuscripts) and more than 50 texts written in Coptic Egyptian estimated to have been hidden in the jar in the period of 400AD although the source of the information goes back much further. Gnostics oversaw the Great or Royal Library of Alexandria, the fantastic depository of ancient texts detailing advanced knowledge and accounts of human history. The Library was dismantled and destroyed in stages over a long period with the death-blow delivered by the Cult-established Roman Church in the period around 415AD. The Church of Rome was the Church of Babylon relocated as I said earlier. Gnostics were not a race. They were a way of perceiving reality. Whenever they established themselves and their information circulated the terrorists of the Church of Rome would target them for destruction. This happened with the Great Library and with the Gnostic Cathars who were burned to death by the psychopaths after a long period of oppression at the siege of the Castle of Monségur in southern France in 1244. The Church has always been terrified of Gnostic information which demolishes the official Christian narrative although there is much in the Bible that supports the Gnostic view if you read it in another way. To anyone studying the texts of what became known as the Nag Hammadi Library it is clear that great swathes of Christian and Biblical belief has its origin with Gnostics sources going back to Sumer. Gnostic themes have been twisted to manipulate the perceived reality of Bible believers. Biblical texts have been in the open for centuries where they could be changed while Gnostic

documents found at Nag Hammadi were sealed away and untouched for 1,600 years. What you see is what they wrote.

Use your *pneuma* not your *nous*

Gnosticism and Gnostic come from 'gnosis' which means knowledge, or rather *secret* knowledge, in the sense of spiritual awareness – knowledge about reality and life itself. The desperation of the Cult's Church of Rome to destroy the Gnostics can be understood when the knowledge they were circulating was the last thing the Cult wanted the population to know. Sixteen hundred years later the same Cult is working hard to undermine and silence me for the same reason. The dynamic between knowledge and ignorance is a constant. 'Time' appears to move on, but essential themes remain the same. We are told to 'use your nous', a Gnostic word for head/brain/intelligence. They said, however, that spiritual awakening or 'salvation' could only be secured by expanding awareness *beyond* what they called *nous* and into *pneuma* or Infinite Self. Obviously as I read these texts the parallels with what I have been saying since 1990 were fascinating to me. There is a universal truth that spans human history and in that case why wouldn't we be talking the same language 16 centuries apart? When you free yourself from the perception program of the five senses and explore expanded realms of consciousness you are going to connect with the same information no matter what the perceived 'era' within a manufactured timeline of a single and tiny range of manipulated frequency. Humans working with 'smart' technology or knocking rocks together in caves is only a timeline appearing to operate within the human frequency band. Expanded awareness and the knowledge it holds have always been there whether the era be Stone Age or computer age. We can only access that knowledge by opening ourselves to its frequency which the five-sense prison cell is designed to stop us doing. Gates, Fauci, Whitty, Vallance, Zuckerberg, Brin, Page, Wojcicki, Bezos, and all the others behind the 'Covid' hoax clearly have a long wait before their range of frequency can make that connection given that an open heart is

crucial to that as we shall see. Instead of accessing knowledge directly through expanded awareness it is given to Cult operatives by the secret society networks of the Cult where it has been passed on over thousands of years outside the public arena. Expanded realms of consciousness is where great artists, composers and writers find their inspiration and where truth awaits anyone open enough to connect with it. We need to go there fast.

Archon hijack

A fifth of the Nag Hammadi texts describe the existence and manipulation of the Archons led by a 'Chief Archon' they call 'Yaldabaoth', or the 'Demiurge', and this is the Christian 'Devil', 'Satan', 'Lucifer', and his demons. Archons in Biblical symbolism are the 'fallen ones' which are also referred to as fallen angels after the angels expelled from heaven according to the Abrahamic religions of Judaism, Christianity and Islam. These angels are claimed to tempt humans to 'sin' ongoing and you will see how accurate that symbolism is during the rest of the book. The theme of 'original sin' is related to the 'Fall' when Adam and Eve were 'tempted by the serpent' and fell from a state of innocence and 'obedience' (connection) with God into a state of disobedience (disconnection). The Fall is said to have brought sin into the world and corrupted everything including human nature. Yaldabaoth, the 'Lord Archon', is described by Gnostics as a 'counterfeit spirit', 'The Blind One', 'The Blind God', and 'The Foolish One'. The Jewish name for Yaldabaoth in Talmudic writings is Samael which translates as 'Poison of God', or 'Blindness of God'. You see the parallels. Yaldabaoth in Islamic belief is the Muslim Jinn devil known as Shaytan – Shaytan is Satan as the same themes are found all over the world in every religion and culture. The 'Lord God' of the Old Testament is the 'Lord Archon' of Gnostic manuscripts and that's why he's such a bloodthirsty bastard. Satan is known by Christians as 'the Demon of Demons' and Gnostics called Yaldabaoth the 'Archon of Archons'. Both are known as 'The Deceiver'. We are talking about the same 'bloke' for sure and these common themes

using different names, storylines and symbolism tell a common tale of the human plight.

Archons are referred to in Nag Hammadi documents as mind parasites, inverters, guards, gatekeepers, detainers, judges, pitiless ones and deceivers. The 'Covid' hoax alone is a glaring example of all these things. The Biblical 'God' is so different in the Old and New Testaments because they are not describing the same phenomenon. The vindictive, angry, hate-filled, 'God' of the Old Testament, known as Yahweh, is Yaldabaoth who is depicted in Cult-dictated popular culture as the 'Dark Lord', 'Lord of Time', Lord (Darth) Vader and Dormammu, the evil ruler of the 'Dark Dimension' trying to take over the 'Earth Dimension' in the Marvel comic movie, *Dr Strange*. Yaldabaoth is both the Old Testament 'god' and the Biblical 'Satan'. Gnostics referred to Yaldabaoth as the 'Great Architect of the Universe'and the Cult-controlled Freemason network calls their god 'the 'Great Architect of the Universe' (also Grand Architect). The 'Great Architect' Yaldabaoth is symbolised by the Cult as the all-seeing eye at the top of the pyramid on the Great Seal of the United States and the dollar bill. Archon is encoded in *arch*-itect as it is in *arch*-angels and *arch*-bishops. All religions have the theme of a force for good and force for evil in some sort of spiritual war and there is a reason for that – the theme is true. The Cult and its non-human masters are quite happy for this to circulate. They present themselves as the force for good fighting evil when they are really the force of evil (absence of love). The whole foundation of Cult modus operandi is inversion. They promote themselves as a force for good and anyone challenging them in pursuit of peace, love, fairness, truth and justice is condemned as a satanic force for evil. This has been the game plan throughout history whether the Church of Rome inquisitions of non-believers or 'conspiracy theorists' and 'anti-vaxxers' of today. The technique is the same whatever the timeline era.

Yaldabaoth is revolting (true)

Yaldabaoth and the Archons are said to have revolted against God with Yaldabaoth claiming to *be* God – the *All That Is*. The Old Testament 'God' (Yaldabaoth) demanded to be worshipped as such: ' *I am* the LORD, and there is none else, there is no God beside me' (Isaiah 45:5). I have quoted in other books a man who said he was the unofficial son of the late Baron Philippe de Rothschild of the Mouton-Rothschild wine producing estates in France who died in 1988 and he told me about the Rothschild 'revolt from God'. The man said he was given the name Phillip Eugene de Rothschild and we shared long correspondence many years ago while he was living under another identity. He said that he was conceived through 'occult incest' which (within the Cult) was 'normal and to be admired'. 'Phillip' told me about his experience attending satanic rituals with rich and famous people whom he names and you can see them and the wider background to Cult Satanism in my other books starting with *The Biggest Secret*. Cult rituals are interactions with Archontic 'gods'. 'Phillip' described Baron Philippe de Rothschild as 'a master Satanist and hater of God' and he used the same term 'revolt from God' associated with Yaldabaoth/Satan/Lucifer/the Devil in describing the Sabbatian Rothschild dynasty. 'I played a key role in my family's revolt from God', he said. That role was to infiltrate in classic Sabbatian style the Christian Church, but eventually he escaped the mind-prison to live another life. The Cult has been targeting religion in a plan to make worship of the Archons the global one-world religion. Infiltration of Satanism into modern 'culture', especially among the young, through music videos, stage shows and other means, is all part of this.

Nag Hammadi texts describe Yaldabaoth and the Archons in their prime form as energy – consciousness – and say they can take form if they choose in the same way that consciousness takes form as a human. Yaldabaoth is called 'formless' and represents a deeply inverted, distorted and chaotic state of consciousness which seeks to attached to humans and turn them into a likeness of itself in an attempt at assimilation. For that to happen it has to manipulate

humans into low frequency mental and emotional states that match its own. Archons can certainly appear in human form and this is the origin of the psychopathic personality. The energetic distortion Gnostics called Yaldabaoth *is* psychopathy. When psychopathic Archons take human form that human will be a psychopath as an expression of Yaldabaoth consciousness. Cult psychopaths are Archons in human form. The principle is the same as that portrayed in the 2009 *Avatar* movie when the American military travelled to a fictional Earth-like moon called Pandora in the Alpha Centauri star system to infiltrate a society of blue people, or Na'vi, by hiding within bodies that looked like the Na'vi. Archons posing as humans have a particular hybrid information field, part human, part Archon, (the ancient 'demigods') which processes information in a way that manifests behaviour to match their psychopathic evil, lack of empathy and compassion, and stops them being influenced by the empathy, compassion and love that a fully-human information field is capable of expressing. Cult bloodlines interbreed, be they royalty or dark suits, for this reason and you have their obsession with incest. Interbreeding with full-blown humans would dilute the Archontic energy field that guarantees psychopathy in its representatives in the human realm.

Gnostic writings say the main non-human forms that Archons take are *serpentine* (what I have called for decades 'reptilian' amid unbounded ridicule from the Archontically-programmed) and what Gnostics describe as 'an unborn baby or foetus with grey skin and dark, unmoving eyes'. This is an excellent representation of the ET 'Greys' of UFO folklore which large numbers of people claim to have seen and been abducted by – Zulu shaman Credo Mutwa among them. I agree with those that believe in extraterrestrial or interdimensional visitations today and for thousands of years past. No wonder with their advanced knowledge and technological capability they were perceived and worshipped as gods for technological and other 'miracles' they appeared to perform. Imagine someone arriving in a culture disconnected from the modern world with a smartphone and computer. They would be

seen as a 'god' capable of 'miracles'. The Renegade Mind, however, wants to know the source of everything and not only the way that source manifests as human or non-human. In the same way that a Renegade Mind seeks the original source material for the 'Covid virus' to see if what is claimed is true. The original source of Archons in form is consciousness – the distorted state of consciousness known to Gnostics as Yaldabaoth.

'Revolt from God' is energetic disconnection

Where I am going next will make a lot of sense of religious texts and ancient legends relating to 'Satan', Lucifer' and the 'gods'. Gnostic descriptions sync perfectly with the themes of my own research over the years in how they describe a consciousness distortion seeking to impose itself on human consciousness. I've referred to the core of infinite awareness in previous books as Infinite Awareness in Awareness of Itself. By that I mean a level of awareness that knows that it is all awareness and is aware of all awareness. From here comes the frequency of love in its true sense and balance which is what love is on one level – the balance of all forces into a single whole called Oneness and Isness. The more we disconnect from this state of love that many call 'God' the constituent parts of that Oneness start to unravel and express themselves as a part and not a whole. They become individualised as intellect, mind, selfishness, hatred, envy, desire for power over others, and such like. This is not a problem in the greater scheme in that 'God', the *All That Is*, can experience all these possibilities through different expressions of itself including humans. What we as expressions of the whole experience the *All That Is* experiences. We are the *All That Is* experiencing itself. As we withdraw from that state of Oneness we disconnect from its influence and things can get very unpleasant and very stupid. Archontic consciousness is at the extreme end of that. It has so disconnected from the influence of Oneness that it has become an inversion of unity and love, an inversion of everything, an inversion of life itself. Evil is appropriately live written backwards. Archontic consciousness is obsessed with death, an inversion of life,

and so its manifestations in Satanism are obsessed with death. They use inverted symbols in their rituals such as the inverted pentagram and cross. Sabbatians as Archontic consciousness incarnate invert Judaism and every other religion and culture they infiltrate. They seek disunity and chaos and they fear unity and harmony as they fear love like garlic to a vampire. As a result the Cult, Archons incarnate, act with such evil, psychopathy and lack of empathy and compassion disconnected as they are from the source of love. How could Bill Gates and the rest of the Archontic psychopaths do what they have to human society in the 'Covid' era with all the death, suffering and destruction involved and have no emotional consequence for the impact on others? Now you know. Why have Zuckerberg, Brin, Page, Wojcicki and company callously censored information warning about the dangers of the 'vaccine' while thousands have been dying and having severe, sometimes life-changing reactions? Now you know. Why have Tedros, Fauci, Whitty, Vallance and their like around the world been using case and death figures they're aware are fraudulent to justify lockdowns and all the deaths and destroyed lives that have come from that? Now you know. Why did Christian Drosten produce and promote a 'testing' protocol that he knew couldn't test for infectious disease which led to a global human catastrophe. Now you know. The Archontic mind doesn't give a shit (Fig 17). I personally think that Gates and major Cult insiders are a form of AI cyborg that the Archons want humans to become.

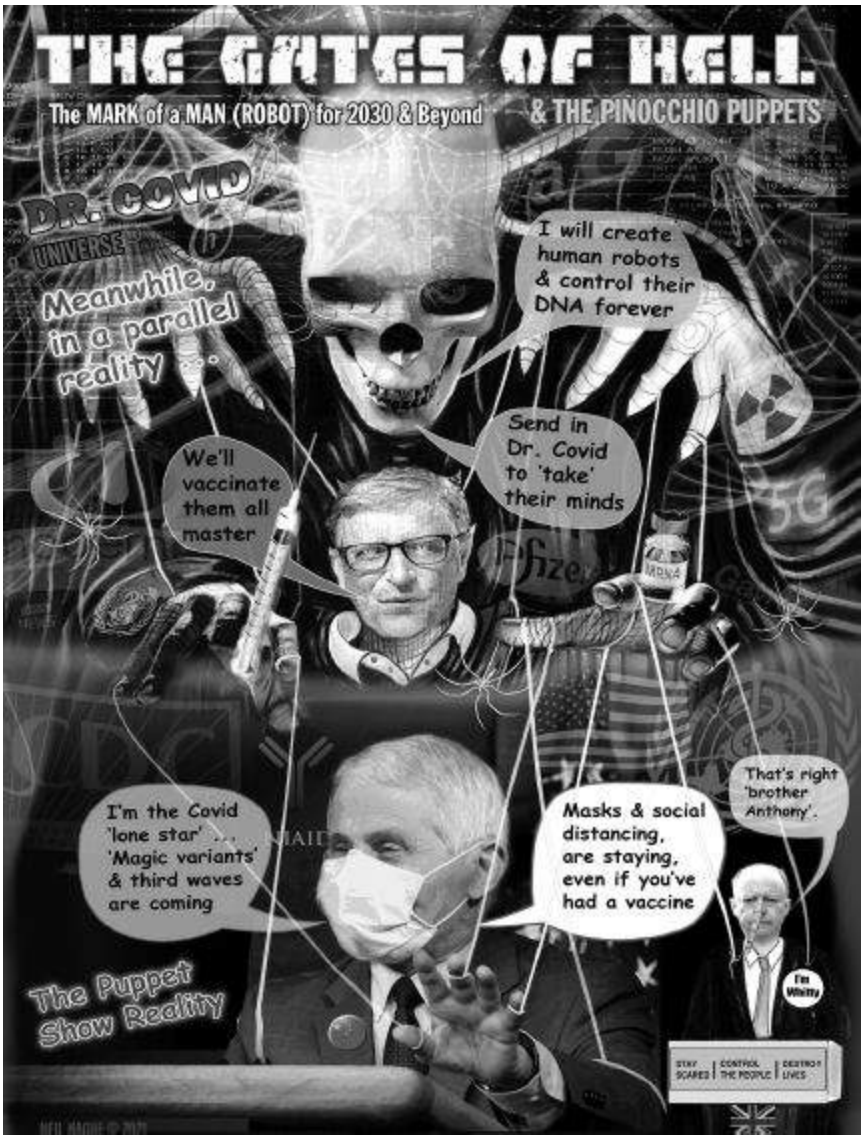

THE GATES OF HELL
The MARK of a MAN (ROBOT) for 2030 & Beyond
& THE PINOCCHIO PUPPETS
DR. COVID
UNIVERSE
Meanwhile, in a parallel reality ...
I will create human robots & control their DNA forever
Send in Dr. Covid to 'take' their minds
We'll vaccinate them all master
5G
I'm the Covid 'lone star' ... 'Magic variants' & third waves are coming
Masks & social distancing, are staying, even if you've had a vaccine
That's right 'brother Anthony'.
The Puppet Show Reality
I'm Whitty
STAY SCARED
CONTROL THE PEOPLE
DESTROY LIVES

Figure 17: Artist Neil Hague's version of the 'Covid' hierarchy.

Human batteries

A state of such inversion does have its consequences, however. The level of disconnection from the Source of All means that you withdraw from that source of energetic sustenance and creativity. This means that you have to find your own supply of energetic power and it has – *us*. When the Morpheus character in the first *Matrix* movie held up a battery he spoke a profound truth when he said: 'The Matrix is a computer-generated dream world built to keep us under control in order to change the human being into one of

these.' The statement was true in all respects. We do live in a technologically-generated virtual reality simulation (more very shortly) and we have been manipulated to be an energy source for Archontic consciousness. The Disney-Pixar animated movie *Monsters, Inc*. in 2001 symbolised the dynamic when monsters in their world had no energy source and they would enter the human world to terrify children in their beds, catch the child's scream, terror (low-vibrational frequencies), and take that energy back to power the monster world. The lead character you might remember was a single giant eye and the symbolism of the Cult's all-seeing eye was obvious. Every thought and emotion is broadcast as a frequency unique to that thought and emotion. Feelings of love and joy, empathy and compassion, are high, quick, frequencies while fear, depression, anxiety, suffering and hate are low, slow, dense frequencies. Which kind do you think Archontic consciousness can connect with and absorb? In such a low and dense frequency state there's no way it can connect with the energy of love and joy. Archons can only feed off energy compatible with their own frequency and they and their Cult agents want to delete the human world of love and joy and manipulate the transmission of low vibrational frequencies through low-vibrational human mental and emotional states. *We are their energy source*. Wars are energetic banquets to the Archons – a world war even more so – and think how much low-frequency mental and emotional energy has been generated from the consequences for humanity of the 'Covid' hoax orchestrated by Archons incarnate like Gates.

The ancient practice of human sacrifice 'to the gods', continued in secret today by the Cult, is based on the same principle. 'The gods' are Archontic consciousness in different forms and the sacrifice is induced into a state of intense terror to generate the energy the Archontic frequency can absorb. Incarnate Archons in the ritual drink the blood which contains an adrenaline they crave which floods into the bloodstream when people are terrorised. Most of the sacrifices, ancient and modern, are children and the theme of 'sacrificing young virgins to the gods' is just code for children. They

have a particular pre-puberty energy that Archons want more than anything and the energy of the young in general is their target. The California Department of Education wants students to chant the names of Aztec gods (Archontic gods) once worshipped in human sacrifice rituals in a curriculum designed to encourage them to 'challenge racist, bigoted, discriminatory, imperialist/colonial beliefs', join 'social movements that struggle for social justice', and 'build new possibilities for a post-racist, post-systemic racism society'. It's the usual Woke crap that inverts racism and calls it anti-racism. In this case solidarity with 'indigenous tribes' is being used as an excuse to chant the names of 'gods' to which people were sacrificed (and still are in secret). What an example of Woke's inability to see beyond black and white, us and them, They condemn the colonisation of these tribal cultures by Europeans (quite right), but those cultures sacrificing people including children to their 'gods', and mass murdering untold numbers as the Aztecs did, is just fine. One chant is to the Aztec god Tezcatlipoca who had a man sacrificed to him in the 5th month of the Aztec calendar. His heart was cut out and he was eaten. Oh, that's okay then. Come on children … after three … Other sacrificial 'gods' for the young to chant their allegiance include Quetzalcoatl, Huitzilopochtli and Xipe Totec. The curriculum says that 'chants, affirmations, and energizers can be used to bring the class together, build unity around ethnic studies principles and values, and to reinvigorate the class following a lesson that may be emotionally taxing or even when student engagement may appear to be low'. Well, that's the cover story, anyway. Chanting and mantras are the repetition of a particular frequency generated from the vocal cords and chanting the names of these Archontic 'gods' tunes you into their frequency. That is the last thing you want when it allows for energetic synchronisation, attachment and perceptual influence. Initiates chant the names of their 'Gods' in their rituals for this very reason.

Vampires of the Woke

Paedophilia is another way that Archons absorb the energy of children. Paedophiles possessed by Archontic consciousness are used as the conduit during sexual abuse for discarnate Archons to vampire the energy of the young they desire so much. Stupendous numbers of children disappear every year never to be seen again although you would never know from the media. Imagine how much low-vibrational energy has been generated by children during the 'Covid' hoax when so many have become depressed and psychologically destroyed to the point of killing themselves. Shocking numbers of children are now taken by the state from loving parents to be handed to others. I can tell you from long experience of researching this since 1996 that many end up with paedophiles and assets of the Cult through corrupt and Cult-owned social services which in the reframing era has hired many psychopaths and emotionless automatons to do the job. Children are even stolen to order using spurious reasons to take them by the corrupt and secret (because they're corrupt) 'family courts'. I have written in detail in other books, starting with *The Biggest Secret* in 1997, about the ubiquitous connections between the political, corporate, government, intelligence and military elites (Cult operatives) and Satanism and paedophilia. If you go deep enough both networks have an interlocking leadership. The Woke mentality has been developed by the Cult for many reasons: To promote almost every aspect of its agenda; to hijack the traditional political left and turn it fascist; to divide and rule; and to target agenda pushbackers. But there are other reasons which relate to what I am describing here. How many happy and joyful Wokers do you ever see especially at the extreme end? They are a mental and psychological mess consumed by emotional stress and constantly emotionally cocked for the next explosion of indignation at someone referring to a female as a female. They are walking, talking, batteries as Morpheus might say emitting frequencies which both enslave them in low-vibrational bubbles of perceptual limitation and feed the Archons. Add to this the hatred claimed to be love; fascism claimed to 'anti-fascism', racism claimed to be 'anti-racism';

exclusion claimed to inclusion; and the abuse-filled Internet trolling. You have a purpose-built Archontic energy system with not a wind turbine in sight and all founded on Archontic *inversion*. We have whole generations now manipulated to serve the Archons with their actions and energy. They will be doing so their entire adult lives unless they snap out of their Archon-induced trance. Is it really a surprise that Cult billionaires and corporations put so much money their way? Where is the energy of joy and laughter, including laughing at yourself which is confirmation of your own emotional security? Mark Twain said: 'The human race has one really effective weapon, and that is laughter.' We must use it all the time. Woke has destroyed comedy because it has no humour, no joy, sense of irony, or self-deprecation. Its energy is dense and intense. *Mmmmm*, lunch says the Archontic frequency. Rudolf Steiner (1861-1925) was the Austrian philosopher and famous esoteric thinker who established Waldorf education or Steiner schools to treat children like unique expressions of consciousness and not minds to be programmed with the perceptions determined by authority. I'd been writing about this energy vampiring for decades when I was sent in 2016 a quote by Steiner. He was spot on:

> There are beings in the spiritual realms for whom anxiety and fear emanating from human beings offer welcome food. When humans have no anxiety and fear, then these creatures starve. If fear and anxiety radiates from people and they break out in panic, then these creatures find welcome nutrition and they become more and more powerful. These beings are hostile towards humanity. Everything that feeds on negative feelings, on anxiety, fear and superstition, despair or doubt, are in reality hostile forces in super-sensible worlds, launching cruel attacks on human beings, while they are being fed ... These are exactly the feelings that belong to contemporary culture and materialism; because it estranges people from the spiritual world, it is especially suited to evoke hopelessness and fear of the unknown in people, thereby calling up the above mentioned hostile forces against them.

Pause for a moment from this perspective and reflect on what has happened in the world since the start of 2020. Not only will pennies drop, but billion dollar bills. We see the same theme from Don Juan Matus, a Yaqui Indian shaman in Mexico and the information source for Peruvian-born writer, Carlos Castaneda, who wrote a series of

books from the 1960s to 1990s. Don Juan described the force manipulating human society and his name for the Archons was the predator:

> We have a predator that came from the depths of the cosmos and took over the rule of our lives. Human beings are its prisoners. The predator is our lord and master. It has rendered us docile, helpless. If we want to protest, it suppresses our protest. If we want to act independently, it demands that we don't do so ... indeed we are held prisoner!
>
> They took us over because we are food to them, and they squeeze us mercilessly because we are their sustenance. Just as we rear chickens in coops, the predators rear us in human coops, humaneros. Therefore, their food is always available to them.
>
> Different cultures, different eras, same recurring theme.

The 'ennoia' dilemma

Nag Hammadi Gnostic manuscripts say that Archon consciousness has no 'ennoia'. This is directly translated as 'intentionality', but I'll use the term 'creative imagination'. The *All That Is* in awareness of itself is the source of all creativity – all possibility – and the more disconnected you are from that source the more you are subsequently denied 'creative imagination'. Given that Archon consciousness is almost entirely disconnected it severely lacks creativity and has to rely on far more mechanical processes of thought and exploit the creative potential of those that do have 'ennoia'. You can see cases of this throughout human society. Archon consciousness almost entirely dominates the global banking system and if we study how that system works you will appreciate what I mean. Banks manifest 'money' out of nothing by issuing lines of 'credit' which is 'money' that has never, does not, and will never exist except in theory. It's a confidence trick. If you think 'credit' figures-on-a-screen 'money' is worth anything you accept it as payment. If you don't then the whole system collapses through lack of confidence in the value of that 'money'. Archontic bankers with no 'ennoia' are 'lending' 'money' that doesn't exist to humans that *do* have creativity – those that have the inspired ideas and create businesses and products. Archon banking feeds off human creativity

which it controls through 'money' creation and debt. Humans have the creativity and Archons exploit that for their own benefit and control while having none themselves. Archon Internet platforms like Facebook claim joint copyright of everything that creative users post and while Archontic minds like Zuckerberg may officially head that company it will be human creatives on the staff that provide the creative inspiration. When you have limitless 'money' you can then buy other companies established by creative humans. Witness the acquisition record of Facebook, Google and their like. Survey the Archon-controlled music industry and you see non-creative dark suit executives making their fortune from the human creativity of their artists. The cases are endless. Research the history of people like Gates and Zuckerberg and how their empires were built on exploiting the creativity of others. Archon minds cannot create out of nothing, but they are skilled (because they have to be) in what Gnostic texts call 'countermimicry'. They can imitate, but not innovate. Sabbatians trawl the creativity of others through backdoors they install in computer systems through their cybersecurity systems. Archon-controlled China is globally infamous for stealing intellectual property and I remember how Hong Kong, now part of China, became notorious for making counterfeit copies of the creativity of others – 'countermimicry'. With the now pervasive and all-seeing surveillance systems able to infiltrate any computer you can appreciate the potential for Archons to vampire the creativity of humans. Author John Lamb Lash wrote in his book about the Nag Hammadi texts, *Not In His Image*:

> Although they cannot originate anything, because they lack the divine factor of ennoia (intentionality), Archons can imitate with a vengeance. Their expertise is simulation (HAL, virtual reality). The Demiurge [Yaldabaoth] fashions a heaven world copied from the fractal patterns [of the original] ... His construction is celestial kitsch, like the fake Italianate villa of a Mafia don complete with militant angels to guard every portal.

This brings us to something that I have been speaking about since the turn of the millennium. Our reality is a simulation; a virtual reality that we think is real. No, I'm not kidding.

Human reality? Well, virtually

I had pondered for years about whether our reality is 'real' or some kind of construct. I remembered being immensely affected on a visit as a small child in the late 1950s to the then newly-opened Planetarium on the Marylebone Road in London which is now closed and part of the adjacent Madame Tussauds wax museum. It was in the middle of the day, but when the lights went out there was the night sky projected in the Planetarium's domed ceiling and it appeared to be so real. The experience never left me and I didn't know why until around the turn of the millennium when I became certain that our 'night sky' and entire reality is a projection, a virtual reality, akin to the illusory world portrayed in the *Matrix* movies. I looked at the sky one day in this period and it appeared to me like the domed roof of the Planetarium. The release of the first *Matrix* movie in 1999 also provided a synchronistic and perfect visual representation of where my mind had been going for a long time. I hadn't come across the Gnostic Nag Hammadi texts then. When I did years later the correlation was once again astounding. As I read Gnostic accounts from 1,600 years and more earlier it was clear that they were describing the same simulation phenomenon. They tell how the Yaldabaoth 'Demiurge' and Archons created a 'bad copy' of original reality to rule over all that were captured by its illusions and the body was a prison to trap consciousness in the 'bad copy' fake reality. Read how Gnostics describe the 'bad copy' and update that to current times and they are referring to what we would call today a virtual reality simulation.

Author John Lamb Lash said 'the Demiurge fashions a heaven world copied from the fractal patterns' of the original through expertise in 'HAL' or virtual reality simulation. Fractal patterns are part of the energetic information construct of our reality, a sort of blueprint. If these patterns were copied in computer terms it would indeed give you a copy of a 'natural' reality in a non-natural frequency and digital form. The principle is the same as making a copy of a website. The original website still exists, but now you can change the copy version to make it whatever you like and it can

become very different to the original website. Archons have done this with our reality, a *synthetic* copy of prime reality that still exists beyond the frequency walls of the simulation. Trapped within the illusions of this synthetic Matrix, however, were and are human consciousness and other expressions of prime reality and this is why the Archons via the Cult are seeking to make the human body synthetic and give us synthetic AI minds to complete the job of turning the entire reality synthetic including what we perceive to be the natural world. To quote Kurzweil: 'Nanobots will infuse all the matter around us with information. Rocks, trees, everything will become these intelligent creatures.' Yes, *synthetic* 'creatures' just as 'Covid' and other genetically-manipulating 'vaccines' are designed to make the human body synthetic. From this perspective it is obvious why Archons and their Cult are so desperate to infuse synthetic material into every human with their 'Covid' scam.

Let there be (electromagnetic) light

Yaldabaoth, the force that created the simulation, or Matrix, makes sense of the Gnostic reference to 'The Great Architect' and its use by Cult Freemasonry as the name of its deity. The designer of the Matrix in the movies is called 'The Architect' and that trilogy is jam-packed with symbolism relating to these subjects. I have contended for years that the angry Old Testament God (Yaldabaoth) is the 'God' being symbolically 'quoted' in the opening of Genesis as 'creating the world'. This is not the creation of prime reality – it's the creation of the *simulation*. The Genesis 'God' says: 'Let there be Light: and there was light.' But what is this 'Light'? I have said for decades that the speed of light (186,000 miles per second) is not the fastest speed possible as claimed by mainstream science and is in fact the frequency walls or outer limits of the Matrix. You can't have a fastest or slowest anything within all possibility when everything is possible. The human body is encoded to operate within the speed of light or *within the simulation* and thus we see only the tiny frequency band of visible *light*. Near-death experiencers who perceive reality outside the body during temporary 'death' describe a very different

form of light and this is supported by the Nag Hammadi texts. Prime reality beyond the simulation ('Upper Aeons' to the Gnostics) is described as a realm of incredible beauty, bliss, love and harmony – a realm of 'watery light' that is so powerful 'there are no shadows'. Our false reality of Archon control, which Gnostics call the 'Lower Aeons', is depicted as a realm with a different kind of 'light' and described in terms of chaos, 'Hell', 'the Abyss' and 'Outer Darkness', where trapped souls are tormented and manipulated by demons (relate that to the 'Covid' hoax alone). The watery light theme can be found in near-death accounts and it is not the same as *simulation* 'light' which is electromagnetic or radiation light within the speed of light – the 'Lower Aeons'. Simulation 'light' is the 'luminous fire' associated by Gnostics with the Archons. The Bible refers to Yaldabaoth as 'that old serpent, called the Devil, and Satan, which deceiveth the whole world' (Revelation 12:9). I think that making a simulated copy of prime reality ('countermimicry') and changing it dramatically while all the time manipulating humanity to believe it to be real could probably meet the criteria of deceiving the whole world. Then we come to the Cult god Lucifer – the *Light Bringer*. Lucifer is symbolic of Yaldabaoth, the bringer of radiation light that forms the bad copy simulation within the speed of light. 'He' is symbolised by the lighted torch held by the Statue of Liberty and in the name 'Illuminati'. Sabbatian-Frankism declares that Lucifer is the true god and Lucifer is the real god of Freemasonry honoured as their 'Great or Grand Architect of the Universe' (simulation).

I would emphasise, too, the way Archontic technologically-generated luminous fire of radiation has deluged our environment since I was a kid in the 1950s and changed the nature of The Field with which we constantly interact. Through that interaction technological radiation is changing us. The Smart Grid is designed to operate with immense levels of communication power with 5G expanding across the world and 6G, 7G, in the process of development. Radiation is the simulation and the Archontic manipulation system. Why wouldn't the Archon Cult wish to unleash radiation upon us to an ever-greater extreme to form

Kurzweil's 'cloud'? The plan for a synthetic human is related to the need to cope with levels of radiation beyond even anything we've seen so far. Biological humans would not survive the scale of radiation they have in their script. The Smart Grid is a technological sub-reality within the technological simulation to further disconnect five-sense perception from expanded consciousness. It's a technological prison of the mind.

Infusing the 'spirit of darkness'

A recurring theme in religion and native cultures is the manipulation of human genetics by a non-human force and most famously recorded as the biblical 'sons of god' (the god*s* plural in the original) who interbred with the daughters of men. The Nag Hammadi *Apocryphon of John* tells the same story this way:

> He [Yaldabaoth] sent his angels [Archons/demons] to the daughters of men, that they might take some of them for themselves and raise offspring for their enjoyment. And at first they did not succeed. When they had no success, they gathered together again and they made a plan together ... And the angels changed themselves in their likeness into the likeness of their mates, filling them with the spirit of darkness, which they had mixed for them, and with evil ... And they took women and begot children out of the darkness according to the likeness of their spirit.

Possession when a discarnate entity takes over a human body is an age-old theme and continues today. It's very real and I've seen it. Satanic and secret society rituals can create an energetic environment in which entities can attach to initiates and I've heard many stories of how people have changed their personality after being initiated even into lower levels of the Freemasons. I have been inside three Freemasonic temples, one at a public open day and two by just walking in when there was no one around to stop me. They were in Ryde, the town where I live, Birmingham, England, when I was with a group, and Boston, Massachusetts. They all felt the same energetically – dark, dense, low-vibrational and sinister. Demonic attachment can happen while the initiate has no idea what is going on. To them it's just a ritual to get in the Masons and do a bit of good

business. In the far more extreme rituals of Satanism human possession is even more powerful and they are designed to make possession possible. The hierarchy of the Cult is dictated by the power and perceived status of the possessing Archon. In this way the Archon hierarchy becomes the Cult hierarchy. Once the entity has attached it can influence perception and behaviour and if it attaches to the extreme then so much of its energy (information) infuses into the body information field that the hologram starts to reflect the nature of the possessing entity. This is the *Exorcist* movie type of possession when facial features change and it's known as shapeshifting. Islam's Jinn are said to be invisible tricksters who change shape, 'whisper', confuse and take human form. These are all traits of the Archons and other versions of the same phenomenon. Extreme possession could certainty infuse the 'spirit of darkness' into a partner during sex as the Nag Hammadi texts appear to describe. Such an infusion can change genetics which is also energetic information. Human genetics is information and the 'spirit of darkness' is information. Mix one with the other and change must happen. Islam has the concept of a 'Jinn baby' through possession of the mother and by Jinn taking human form. There are many ways that human genetics can be changed and remember that Archons have been aware all along of advanced techniques to do this. What is being done in human society today – and far more – was known about by Archons at the time of the 'fallen ones' and their other versions described in religions and cultures.

Archons and their human-world Cult are obsessed with genetics as we see today and they know this dictates how information is processed into perceived reality during a human life. They needed to produce a human form that would decode the simulation and this is symbolically known as 'Adam and Eve' who left the 'garden' (prime reality) and 'fell' into Matrix reality. The simulation is not a 'physical' construct (there is no 'physical'); it is a source of information. Think Wi-Fi again. The simulation is an energetic field encoded with information and body-brain systems are designed to decode that information encoded in wave or frequency form which

is transmitted to the brain as electrical signals. These are decoded by the brain to construct our sense of reality – an illusory 'physical' world that only exists in the brain or the mind. Virtual reality games mimic this process using the same sensory decoding system. Information is fed to the senses to decode a virtual reality that can appear so real, but isn't (Figs 18 and 19). Some scientists believe – and I agree with them – that what we perceive as 'physical' reality only exists when we are looking or observing. The act of perception or focus triggers the decoding systems which turn waveform information into holographic reality. When we are not observing something our reality reverts from a holographic state to a waveform state. This relates to the same principle as a falling tree not making a noise unless someone is there to hear it or decode it. The concept makes sense from the simulation perspective. A computer is not decoding all the information in a Wi-Fi field all the time and only decodes or brings into reality on the screen that part of Wi-Fi that it's decoding – focusing upon – at that moment.

Figure 18: Virtual reality technology 'hacks' into the body's five-sense decoding system.

Figure 19: The result can be experienced as very 'real'.

Interestingly, Professor Donald Hoffman at the Department of Cognitive Sciences at the University of California, Irvine, says that our experienced reality is like a computer interface that shows us only the level with which we interact while hiding all that exists beyond it: 'Evolution shaped us with a user interface that hides the truth. Nothing that we see is the truth – the very language of space and time and objects is the wrong language to describe reality.' He is correct in what he says on so many levels. Space and time are not a universal reality. They are a phenomenon of decoded *simulation* reality as part of the process of enslaving our sense of reality. Near-death experiencers report again and again how space and time did not exist as we perceive them once they were free of the body – body decoding systems. You can appreciate from this why Archons and their Cult are so desperate to entrap human attention in the five senses where we are in the Matrix and of the Matrix. Opening your mind to expanded states of awareness takes you beyond the information confines of the simulation and you become aware of knowledge and insights denied to you before. This is what we call 'awakening' – *awakening from the Matrix* – and in the final chapter I will relate this to current events.

Where are the 'aliens'?

A simulation would explain the so-called 'Fermi Paradox' named after Italian physicist Enrico Fermi (1901-1954) who created the first nuclear reactor. He considered the question of why there is such a lack of extraterrestrial activity when there are so many stars and planets in an apparently vast universe; but what if the night sky that we see, or think we do, is a simulated projection as I say? If you control the simulation and your aim is to hold humanity fast in essential ignorance would you want other forms of life including advanced life coming and going sharing information with humanity? Or would you want them to believe they were isolated and apparently alone? Themes of human isolation and apartness are common whether they be the perception of a lifeless universe or the fascist isolation laws of the 'Covid' era. Paradoxically the very

existence of a simulation means that we are not alone when some force had to construct it. My view is that experiences that people have reported all over the world for centuries with Reptilians and Grey entities are Archon phenomena as Nag Hammadi texts describe; and that benevolent 'alien' interactions are non-human groups that come in and out of the simulation by overcoming Archon attempts to keep them out. It should be highlighted, too, that Reptilians and Greys are obsessed with *genetics* and *technology* as related by cultural accounts and those who say they have been abducted by them. Technology is their way of overcoming some of the limitations in their creative potential and our technology-driven and controlled human society of today is *arch*etypical Archon-Reptilian-Grey modus operandi. Technocracy is really *Archon*tocracy. The Universe does not have to be as big as it appears with a simulation. There is no space or distance only information decoded into holographic reality. What we call 'space' is only the absence of holographic 'objects' and that 'space' is The Field of energetic information which connects everything into a single whole. The same applies with the artificially-generated information field of the simulation. The Universe is not big or small as a physical reality. It is decoded information, that's all, and its perceived size is decided by the way the simulation is encoded to make it appear. The entire night sky as we perceive it only exists in our brain and so where are those 'millions of light years'? The 'stars' on the ceiling of the Planetarium looked a vast distance away.

There's another point to mention about 'aliens'. I have been highlighting since the 1990s the plan to stage a fake 'alien invasion' to justify the centralisation of global power and a world military. Nazi scientist Werner von Braun, who was taken to America by Operation Paperclip after World War Two to help found NASA, told his American assistant Dr Carol Rosin about the Cult agenda when he knew he was dying in 1977. Rosin said that he told her about a sequence that would lead to total human control by a one-world government. This included threats from terrorism, rogue nations, meteors and asteroids before finally an 'alien invasion'. All of these

things, von Braun said, would be bogus and what I would refer to as a No-Problem-Reaction-Solution. Keep this in mind when 'the aliens are coming' is the new mantra. The aliens are not coming – they are *already here* and they have infiltrated human society while looking human. French-Canadian investigative journalist Serge Monast said in 1994 that he had uncovered a NASA/military operation called Project Blue Beam which fits with what Werner von Braun predicted. Monast died of a 'heart attack' in 1996 the day after he was arrested and spent a night in prison. He was 51. He said Blue Beam was a plan to stage an alien invasion that would include religious figures beamed holographically into the sky as part of a global manipulation to usher in a 'new age' of worshipping what I would say is the Cult 'god' Yaldabaoth in a one-world religion. Fake holographic asteroids are also said to be part of the plan which again syncs with von Braun. How could you stage an illusory threat from asteroids unless they were holographic inserts? This is pretty straightforward given the advanced technology outside the public arena and the fact that our 'physical' reality is holographic anyway. Information fields would be projected and we would decode them into the illusion of a 'physical' asteroid. If they can sell a global 'pandemic' with a 'virus' that doesn't exist what will humans not believe if government and media tell them?

All this is particularly relevant as I write with the Pentagon planning to release in June, 2021, information about 'UFO sightings'. I have been following the UFO story since the early 1990s and the common theme throughout has been government and military denials and cover up. More recently, however, the Pentagon has suddenly become more talkative and apparently open with Air Force pilot radar images released of unexplained craft moving and changing direction at speeds well beyond anything believed possible with human technology. Then, in March, 2021, former Director of National Intelligence John Ratcliffe said a Pentagon report months later in June would reveal a great deal of information about UFO sightings unknown to the public. He said the report would have 'massive implications'. The order to do this was included bizarrely

in a $2.3 trillion 'coronavirus' relief and government funding bill passed by the Trump administration at the end of 2020. I would add some serious notes of caution here. I have been pointing out since the 1990s that the US military and intelligence networks have long had craft – 'flying saucers' or anti-gravity craft – which any observer would take to be extraterrestrial in origin. Keeping this knowledge from the public allows craft flown by *humans* to be perceived as alien visitations. I am not saying that 'aliens' do not exist. I would be the last one to say that, but we have to be streetwise here. President Ronald Reagan told the UN General Assembly in 1987: 'I occasionally think how quickly our differences worldwide would vanish if we were facing an alien threat from outside this world.' That's the idea. Unite against a common 'enemy' with a common purpose behind your 'saviour force' (the Cult) as this age-old technique of mass manipulation goes global.

Science moves this way …

I could find only one other person who was discussing the simulation hypothesis publicly when I concluded it was real. This was Nick Bostrom, a Swedish-born philosopher at the University of Oxford, who has explored for many years the possibility that human reality is a computer simulation although his version and mine are not the same. Today the simulation and holographic reality hypothesis have increasingly entered the scientific mainstream. Well, the more open-minded mainstream, that is. Here are a few of the ever-gathering examples. American nuclear physicist Silas Beane led a team of physicists at the University of Bonn in Germany pursuing the question of whether we live in a simulation. They concluded that we probably do and it was likely based on a lattice of cubes. They found that cosmic rays align with that specific pattern. The team highlighted the Greisen–Zatsepin–Kuzmin (GZK) limit which refers to cosmic ray particle interaction with cosmic background radiation that creates an apparent boundary for cosmic ray particles. They say in a paper entitled 'Constraints on the Universe as a Numerical Simulation' that this 'pattern of constraint' is exactly what you

would find with a computer simulation. They also made the point that a simulation would create its own 'laws of physics' that would limit possibility. I've been making the same point for decades that the *perceived* laws of physics relate only to this reality, or what I would later call the simulation. When designers write codes to create computer and virtual reality games they are the equivalent of the laws of physics for that game. Players interact within the limitations laid out by the coding. In the same way those who wrote the codes for the simulation decided the laws of physics that would apply. These can be overridden by expanded states of consciousness, but not by those enslaved in only five-sense awareness where simulation codes rule. Overriding the codes is what people call 'miracles'. They are not. They are bypassing the encoded limits of the simulation. A population caught in simulation perception would have no idea that this was their plight. As the Bonn paper said: 'Like a prisoner in a pitch-black cell we would not be able to see the "walls" of our prison,' That's true if people remain mesmerised by the five senses. Open to expanded awareness and those walls become very clear. The main one is the speed of light.

American theoretical physicist James Gates is another who has explored the simulation question and found considerable evidence to support the idea. Gates was Professor of Physics at the University of Maryland, Director of The Center for String and Particle Theory, and on Barack Obama's Council of Advisors on Science and Technology. He and his team found *computer codes* of digital data embedded in the fabric of our reality. They relate to on-off electrical charges of 1 and 0 in the binary system used by computers. 'We have no idea what they are doing there', Gates said. They found within the energetic fabric mathematical sequences known as error-correcting codes or block codes that 'reboot' data to its original state or 'default settings' when something knocks it out of sync. Gates was asked if he had found a set of equations embedded in our reality indistinguishable from those that drive search engines and browsers and he said: 'That is correct.' Rich Terrile, director of the Centre for Evolutionary Computation and Automated Design at NASA's Jet

Propulsion Laboratory, has said publicly that he believes the Universe is a digital hologram that must have been created by a form of intelligence. I agree with that in every way. Waveform information is delivered electrically by the senses to the brain which constructs a *digital* holographic reality that we call the 'world'. This digital level of reality can be read by the esoteric art of numerology. Digital holograms are at the cutting edge of holographics today. We have digital technology everywhere designed to access and manipulate our digital level of perceived reality. Synthetic mRNA in 'Covid vaccines' has a digital component to manipulate the body's digital 'operating system'.

Reality is numbers

How many know that our reality can be broken down to numbers and codes that are the same as computer games? Max Tegmark, a physicist at the Massachusetts Institute of Technology (MIT), is the author of *Our Mathematical Universe* in which he lays out how reality can be entirely described by numbers and maths in the way that a video game is encoded with the 'physics' of computer games. Our world and computer virtual reality are essentially the same. Tegmark imagines the perceptions of characters in an advanced computer game when the graphics are so good they don't know they are in a game. They think they can bump into real objects (electromagnetic resistance in our reality), fall in love and feel emotions like excitement. When they began to study the apparently 'physical world' of the video game they would realise that everything was made of pixels (which have been found in our energetic reality as must be the case when on one level our world is digital). What computer game characters thought was physical 'stuff', Tegmark said, could actually be broken down into numbers:

> And we're exactly in this situation in our world. We look around and it doesn't seem that mathematical at all, but everything we see is made out of elementary particles like quarks and electrons. And what properties does an electron have? Does it have a smell or a colour or a texture? No! ... We physicists have come up with geeky names for [Electron] properties, like

electric charge, or spin, or lepton number, but the electron doesn't care what we call it, the properties are just numbers.

This is the illusory reality Gnostics were describing. This is the simulation. The A, C, G, and T codes of DNA have a binary value – A and C = 0 while G and T = 1. This has to be when the simulation is digital and the body must be digital to interact with it. Recurring mathematical sequences are encoded throughout reality and the body. They include the Fibonacci sequence in which the two previous numbers are added to get the next one, as in ... 1, 1, 2, 3, 5, 8, 13, 21, 34, 55, etc. The sequence is encoded in the human face and body, proportions of animals, DNA, seed heads, pine cones, trees, shells, spiral galaxies, hurricanes and the number of petals in a flower. The list goes on and on. There are fractal patterns – a 'never-ending pattern that is infinitely complex and self-similar across all scales in the as above, so below, principle of holograms. These and other famous recurring geometrical and mathematical sequences such as Phi, Pi, Golden Mean, Golden Ratio and Golden Section are *computer codes* of the simulation. I had to laugh and give my head a shake the day I finished this book and it went into the production stage. I was sent an article in *Scientific American* published in April, 2021, with the headline 'Confirmed! We Live in a Simulation'. Two decades after I first said our reality is a simulation and the speed of light is it's outer limit the article suggested that we do live in a simulation and that the speed of light is its outer limit. I left school at 15 and never passed a major exam in my life while the writer was up to his eyes in qualifications. As I will explain in the final chapter *knowing* is far better than thinking and they come from very different sources. The article rightly connected the speed of light to the processing speed of the 'Matrix' and said what has been in my books all this time … 'If we are in a simulation, as it appears, then space is an abstract property written in code. It is not real'. No it's not and if we live in a simulation something created it and it wasn't *us*. 'That David Icke says we are manipulated by aliens' – he's crackers.'

Wow …

The reality that humanity thinks is so real is an illusion. Politicians, governments, scientists, doctors, academics, law enforcement, media, school and university curriculums, on and on, are all founded on a world that *does not exist* except as a simulated prison cell. Is it such a stretch to accept that 'Covid' doesn't exist when our entire 'physical' reality doesn't exist? Revealed here is the knowledge kept under raps in the Cult networks of compartmentalised secrecy to control humanity's sense of reality by inducing the population to believe in a reality that's not real. If it wasn't so tragic in its experiential consequences the whole thing would be hysterically funny. None of this is new to Renegade Minds. Ancient Greek philosopher Plato (about 428 to about 347BC) was a major influence on Gnostic belief and he described the human plight thousands of years ago with his Allegory of the Cave. He told the symbolic story of prisoners living in a cave who had never been outside. They were chained and could only see one wall of the cave while behind them was a fire that they could not see. Figures walked past the fire casting shadows on the prisoners' wall and those moving shadows became their sense of reality. Some prisoners began to study the shadows and were considered experts on them (today's academics and scientists), but what they studied was only an illusion (today's academics and scientists). A prisoner escaped from the cave and saw reality as it really is. When he returned to report this revelation they didn't believe him, called him mad and threatened to kill him if he tried to set them free. Plato's tale is not only a brilliant analogy of the human plight and our illusory reality. It describes, too, the dynamics of the 'Covid' hoax. I have only skimmed the surface of these subjects here. The aim of this book is to crisply connect all essential dots to put what is happening today into its true context. All subject areas and their connections in this chapter are covered in great evidential detail in *Everything You Need To Know, But Have Never Been Told* and *The Answer*.

They say that bewildered people 'can't see the forest for the trees'. Humanity, however, can't see the forest for the *twigs*. The five senses

see only twigs while Renegade Minds can see the forest and it's the forest where the answers lie with the connections that reveals. Breaking free of perceptual programming so the forest can be seen is the way we turn all this around. Not breaking free is how humanity got into this mess. The situation may seem hopeless, but I promise you it's not. We are a perceptual heartbeat from paradise if only we knew.

CHAPTER TWELVE

Escaping Wetiko

Life is simply a vacation from the infinite
Dean Cavanagh

Renegade Minds weave the web of life and events and see common themes in the apparently random. They are always there if you look for them and their pursuit is aided by incredible synchronicity that comes when your mind is open rather than mesmerised by what it thinks it can see.

Infinite awareness is infinite possibility and the more of infinite possibility that we access the more becomes infinitely possible. That may be stating the apparently obvious, but it is a devastatingly-powerful fact that can set us free. We are a point of attention within an infinity of consciousness. The question is how much of that infinity do we choose to access? How much knowledge, insight, awareness, wisdom, do we want to connect with and explore? If your focus is only in the five senses you will be influenced by a fraction of infinite awareness. I mean a range so tiny that it gives new meaning to infinitesimal. Limitation of self-identity and a sense of the possible limit accordingly your range of consciousness. We are what we think we are. Life is what we think it is. The dream is the dreamer and the dreamer is the dream. Buddhist philosophy puts it this way: 'As a thing is viewed, so it appears.' Most humans live in the realm of touch, taste, see, hear, and smell and that's the limit of their sense of the possible and sense of self. Many will follow a religion and speak of a God in his heaven, but their lives are still

dominated by the five senses in their perceptions and actions. The five senses become the arbiter of everything. When that happens all except a smear of infinity is sealed away from influence by the rigid, unyielding, reality bubbles that are the five-sense human or Phantom Self. Archon Cult methodology is to isolate consciousness within five-sense reality – the simulation – and then program that consciousness with a sense of self and the world through a deluge of life-long information designed to instil the desired perception that allows global control. Efforts to do this have increased dramatically with identity politics as identity bubbles are squeezed into the minutiae of five-sense detail which disconnect people even more profoundly from the infinite 'I'.

Five-sense focus and self-identity are like a firewall that limits access to the infinite realms. You only perceive one radio or television station and no other. We'll take that literally for a moment. Imagine a vast array of stations giving different information and angles on reality, but you only ever listen to one. Here we have the human plight in which the population is overwhelmingly confined to CultFM. This relates only to the frequency range of CultFM and limits perception and insight to that band – limits *possibility* to that band. It means you are connecting with an almost imperceptibly minuscule range of possibility and creative potential within the infinite Field. It's a world where everything seems apart from everything else and where synchronicity is rare. Synchronicity is defined in the dictionary as 'the happening by chance of two or more related or similar events at the same time'. Use of 'by chance' betrays a complete misunderstanding of reality. Synchronicity is not 'by chance'. As people open their minds, or 'awaken' to use the term, they notice more and more coincidences in their lives, bits of 'luck', apparently miraculous happenings that put them in the right place at the right time with the right people. Days become peppered with 'fancy meeting you here' and 'what are the chances of that?' My entire life has been lived like this and ever more so since my own colossal awakening in 1990 and 91 which transformed my sense of reality. Synchronicity is not 'by chance'; it is by accessing expanded

realms of possibility which allow expanded potential for manifestation. People broadcasting the same vibe from the same openness of mind tend to be drawn 'by chance' to each other through what I call frequency magnetism and it's not only people. In the last more than 30 years incredible synchronicity has also led me through the Cult maze to information in so many forms and to crucial personal experiences. These 'coincidences' have allowed me to put the puzzle pieces together across an enormous array of subjects and situations. Those who have breached the bubble of five-sense reality will know exactly what I mean and this escape from the perceptual prison cell is open to everyone whenever they make that choice. This may appear super-human when compared with the limitations of 'human', but it's really our natural state. 'Human' as currently experienced is consciousness in an unnatural state of induced separation from the infinity of the whole. I'll come to how this transformation into unity can be made when I have described in more detail the force that holds humanity in servitude by denying this access to infinite self.

The Wetiko factor

I have been talking and writing for decades about the way five-sense mind is systematically barricaded from expanded awareness. I have used the analogy of a computer (five-sense mind) and someone at the keyboard (expanded awareness). Interaction between the computer and the operator is symbolic of the interaction between five-sense mind and expanded awareness. The computer directly experiences the Internet and the operator experiences the Internet via the computer which is how it's supposed to be – the two working as one. Archons seek to control that point where the operator connects with the computer to stop that interaction (Fig 20). Now the operator is banging the keyboard and clicking the mouse, but the computer is not responding and this happens when the computer is taken over – *possessed* – by an appropriately-named computer 'virus'. The operator has lost all influence over the computer which goes its own way making decisions under the control of the 'virus'. I have

just described the dynamic through which the force known to Gnostics as Yaldabaoth and Archons disconnects five-sense mind from expanded awareness to imprison humanity in perceptual servitude.

Figure 20: The mind 'virus' I have been writing about for decades seeks to isolate five-sense mind (the computer) from the true 'I'. (Image by Neil Hague).

About a year ago I came across a Native American concept of Wetiko which describes precisely the same phenomenon. Wetiko is the spelling used by the Cree and there are other versions including wintiko and windigo used by other tribal groups. They spell the name with lower case, but I see Wetiko as a proper noun as with Archons and prefer a capital. I first saw an article about Wetiko by writer and researcher Paul Levy which so synced with what I had been writing about the computer/operator disconnection and later the Archons. I then read his book, the fascinating *Dispelling Wetiko, Breaking the Spell of Evil*. The parallels between what I had concluded long before and the Native American concept of Wetiko were so clear and obvious that it was almost funny. For Wetiko see the Gnostic Archons for sure and the Jinn, the Predators, and every other name for a force of evil, inversion and chaos. Wetiko is the Native American name for the force that divides the computer from

the operator (Fig 21). Indigenous author Jack D. Forbes, a founder of the Native American movement in the 1960s, wrote another book about Wetiko entitled *Columbus And Other Cannibals – The Wetiko Disease of Exploitation, Imperialism, and Terrorism* which I also read. Forbes says that Wetiko refers to an evil person or spirit 'who terrorizes other creatures by means of terrible acts, including cannibalism'. Zulu shaman Credo Mutwa told me that African accounts tell how cannibalism was brought into the world by the Chitauri 'gods' – another manifestation of Wetiko. The distinction between 'evil person or spirit' relates to Archons/Wetiko possessing a human or acting as pure consciousness. Wetiko is said to be a sickness of the soul or spirit and a state of being that takes but gives nothing back – the Cult and its operatives perfectly described. Black Hawk, a Native American war leader defending their lands from confiscation, said European invaders had 'poisoned hearts' – Wetiko hearts – and that this would spread to native societies. Mention of the heart is very significant as we shall shortly see. Forbes writes: 'Tragically, the history of the world for the past 2,000 years is, in great part, the story of the epidemiology of the wetiko disease.' Yes, and much longer. Forbes is correct when he says: 'The wetikos destroyed Egypt and Babylon and Athens and Rome and Tenochtitlan [capital of the Aztec empire] and perhaps now they will destroy the entire earth.' Evil, he said, is the number one export of a Wetiko culture – see its globalisation with 'Covid'. Constant war, mass murder, suffering of all kinds, child abuse, Satanism, torture and human sacrifice are all expressions of Wetiko and the Wetiko possessed. The world is Wetiko made manifest, *but it doesn't have to be*. There is a way out of this even now.

Figure 21: The mind 'virus' is known to Native Americans as 'Wetiko'. (Image by Neil Hague).

Cult of Wetiko

Wetiko is the Yaldabaoth frequency distortion that seeks to attach to human consciousness and absorb it into its own. Once this connection is made Wetiko can drive the perceptions of the target which they believe to be coming from their own mind. All the horrors of history and today from mass killers to Satanists, paedophiles like Jeffrey Epstein and other psychopaths, are the embodiment of Wetiko and express its state of being in all its grotesqueness. The Cult is Wetiko incarnate, Yaldabaoth incarnate, and it seeks to facilitate Wetiko assimilation of humanity in totality into its distortion by manipulating the population into low frequency states that match its own. Paul Levy writes: 'Holographically enforced within the psyche of every human being the wetiko virus pervades and underlies the entire field of consciousness, and can therefore potentially manifest through any one of us at any moment if we are not mindful.' The 'Covid' hoax has achieved this with many people, but others have not fallen into Wetiko's frequency lair. Players in the 'Covid' human catastrophe including Gates, Schwab, Tedros, Fauci, Whitty, Vallance, Johnson, Hancock, Ferguson, Drosten, and all the rest, including the psychopath psychologists, are expressions of Wetiko. This is why

they have no compassion or empathy and no emotional consequence for what they do that would make them stop doing it. Observe all the people who support the psychopaths in authority against the Pushbackers despite the damaging impact the psychopaths have on their own lives and their family's lives. You are again looking at Wetiko possession which prevents them seeing through the lies to the obvious scam going on. *Why can't they see it?* Wetiko won't let them see it. The perceptual divide that has now become a chasm is between the Wetikoed and the non-Wetikoed.

Paul Levy describes Wetiko in the same way that I have long described the Archontic force. They are the same distorted consciousness operating across dimensions of reality: '… the subtle body of wetiko is not located in the third dimension of space and time, literally existing in another dimension … it is able to affect ordinary lives by mysteriously interpenetrating into our three-dimensional world.' Wetiko does this through its incarnate representatives in the Cult and by weaving itself into The Field which on our level of reality is the electromagnetic information field of the simulation or Matrix. More than that, the simulation *is* Wetiko / Yaldabaoth. Caleb Scharf, Director of Astrobiology at Columbia University, has speculated that 'alien life' could be so advanced that it has transcribed itself into the quantum realm to become what we call physics. He said intelligence indistinguishable from the fabric of the Universe would solve many of its greatest mysteries:

> Perhaps hyper-advanced life isn't just external. Perhaps it's already all around. It is embedded in what we perceive to be physics itself, from the root behaviour of particles and fields to the phenomena of complexity and emergence ... In other words, life might not just be in the equations. It might BE the equations [My emphasis].

Scharf said it is possible that 'we don't recognise advanced life because it forms an integral and unsuspicious part of what we've considered to be the natural world'. I agree. Wetiko/Yaldabaoth *is* the simulation. We are literally in the body of the beast. But that doesn't mean it has to control us. We all have the power to overcome Wetiko

influence and the Cult knows that. I doubt it sleeps too well because it knows that.

Which Field?

This, I suggest, is how it all works. There are two Fields. One is the fierce electromagnetic light of the Matrix within the speed of light; the other is the 'watery light' of The Field beyond the walls of the Matrix that connects with the Great Infinity. Five-sense mind and the decoding systems of the body attach us to the Field of Matrix light. They have to or we could not experience this reality. Five-sense mind sees only the Matrix Field of information while our expanded consciousness is part of the Infinity Field. When we open our minds, and most importantly our hearts, to the Infinity Field we have a mission control which gives us an expanded perspective, a road map, to understand the nature of the five-sense world. If we are isolated only in five-sense mind there is no mission control. We're on our own trying to understand a world that's constantly feeding us information to ensure we do not understand. People in this state can feel 'lost' and bewildered with no direction or radar. You can see ever more clearly those who are influenced by the Fields of Big Infinity or little five-sense mind simply by their views and behaviour with regard to the 'Covid' hoax. We have had this division throughout known human history with the mass of the people on one side and individuals who could see and intuit beyond the walls of the simulation – Plato's prisoner who broke out of the cave and saw reality for what it is. Such people have always been targeted by Wetiko/Archon-possessed authority, burned at the stake or demonised as mad, bad and dangerous. The Cult today and its global network of 'anti-hate', 'anti-fascist' Woke groups are all expressions of Wetiko attacking those exposing the conspiracy, 'Covid' lies and the 'vaccine' agenda.

Woke as a whole is Wetiko which explains its black and white mentality and how at one it is with the Wetiko-possessed Cult. Paul Levy said: 'To be in this paradigm is to still be under the thrall of a two-valued logic – where things are either true or false – of a

wetikoized mind.' Wetiko consciousness is in a permanent rage, therefore so is Woke, and then there is Woke inversion and contradiction. 'Anti-fascists' act like fascists because fascists *and* 'anti-fascists' are both Wetiko at work. Political parties act the same while claiming to be different for the same reason. Secret society and satanic rituals are attaching initiates to Wetiko and the cold, ruthless, psychopathic mentality that secures the positions of power all over the world is Wetiko. Reframing 'training programmes' have the same cumulative effect of attaching Wetiko and we have their graduates described as automatons and robots with a cold, psychopathic, uncaring demeanour. They are all traits of Wetiko possession and look how many times they have been described in this book and elsewhere with regard to personnel behind 'Covid' including the police and medical profession. Climbing the greasy pole in any profession in a Wetiko society requires traits of Wetiko to get there and that is particularly true of politics which is not about fair competition and pre-eminence of ideas. It is founded on how many backs you can stab and arses you can lick. This culminated in the global 'Covid' coordination between the Wetiko possessed who pulled it off in all the different countries without a trace of empathy and compassion for their impact on humans. Our sight sense can see only holographic form and not the Field which connects holographic form. Therefore we perceive 'physical' objects with 'space' in between. In fact that 'space' is energy/consciousness operating on multiple frequencies. One of them is Wetiko and that connects the Cult psychopaths, those who submit to the psychopaths, and those who serve the psychopaths in the media operations of the world. Wetiko is Gates. Wetiko is the mask-wearing submissive. Wetiko is the fake journalist and 'fact-checker'. The Wetiko Field is coordinating the whole thing. Psychopaths, gofers, media operatives, 'anti-hate' hate groups, 'fact-checkers' and submissive people work as one unit *even without human coordination* because they are attached to the *same* Field which is organising it all (Fig 22). Paul Levy is here describing how Wetiko-possessed people are drawn together and refuse to let any information breach their rigid

perceptions. He was writing long before 'Covid', but I think you will recognise followers of the 'Covid' religion *oh just a little bit*:

People who are channelling the vibratory frequency of wetiko align with each other through psychic resonance to reinforce their unspoken shared agreement so as to uphold their deranged view of reality. Once an unconscious content takes possession of certain individuals, it irresistibly draws them together by mutual attraction and knits them into groups tied together by their shared madness that can easily swell into an avalanche of insanity.

A psychic epidemic is a closed system, which is to say that it is insular and not open to any new information or informing influences from the outside world which contradict its fixed, limited, and limiting perspective.

There we have the Woke mind and the 'Covid' mind. Compatible resonance draws the awakening together, too, which is clearly happening today.

Figure 22: The Wetiko Field from which the Cult pyramid and its personnel are made manifest. (Image by Neil Hague).

Spiritual servitude

Wetiko doesn't care about humans. It's not human; it just possesses humans for its own ends and the effect (depending on the scale of

possession) can be anything from extreme psychopathy to unquestioning obedience. Wetiko's worst nightmare is for human consciousness to expand beyond the simulation. Everything is focussed on stopping that happening through control of information, thus perception, thus frequency. The 'education system', media, science, medicine, academia, are all geared to maintaining humanity in five-sense servitude as is the constant stimulation of low-vibrational mental and emotional states (see 'Covid'). Wetiko seeks to dominate those subconscious spaces between five-sense perception and expanded consciousness where the computer meets the operator. From these subconscious hiding places Wetiko speaks to us to trigger urges and desires that we take to be our own and manipulate us into anything from low-vibrational to psychopathic states. Remember how Islam describes the Jinn as invisible tricksters that 'whisper' and confuse. Wetiko is the origin of the 'trickster god' theme that you find in cultures all over the world. Jinn, like the Archons, are Wetiko which is terrified of humans awakening and reconnecting with our true self for then its energy source has gone. With that the feedback loop breaks between Wetiko and human perception that provides the energetic momentum on which its very existence depends as a force of evil. Humans are both its target and its source of survival, but only if we are operating in low-vibrational states of fear, hate, depression and the background anxiety that most people suffer. We are Wetiko's target because we are its key to survival. It needs us, not the other way round. Paul Levy writes:

A vampire has no intrinsic, independent, substantial existence in its own right; it only exists in relation to us. The pathogenic, vampiric mind-parasite called wetiko is nothing in itself – not being able to exist from its own side – yet it has a 'virtual reality' such that it can potentially destroy our species …

…The fact that a vampire is not reflected by a mirror can also mean that what we need to see is that there's nothing, no-thing to see, other than ourselves. The fact that wetiko is the expression of something inside of us means that the cure for wetiko is with us as well. The critical issue is finding this cure within us and then putting it into effect.

Evil begets evil because if evil does not constantly expand and find new sources of energetic sustenance its evil, its *distortion*, dies with the assimilation into balance and harmony. Love is the garlic to Wetiko's vampire. Evil, the absence of love, cannot exist in the presence of love. I think I see a way out of here. I have emphasised so many times over the decades that the Archons/Wetiko and their Cult are not all powerful. *They are not*. I don't care how it looks even now *they are not*. I have not called them little boys in short trousers for effect. I have said it because it is true. Wetiko's insatiable desire for power over others is not a sign of its omnipotence, but its insecurity. Paul Levy writes: 'Due to the primal fear which ultimately drives it and which it is driven to cultivate, wetiko's body politic has an intrinsic and insistent need for centralising power and control so as to create imagined safety for itself.' *Yeeeeeees!* Exactly! Why does Wetiko want humans in an ongoing state of fear? Wetiko itself *is* fear and it is petrified of love. As evil is an absence of love, so love is an absence of fear. Love conquers all and *especially* Wetiko which *is* fear. Wetiko brought fear into the world when it wasn't here before. *Fear* was the 'fall', the fall into low-frequency ignorance and illusion – fear is **F**alse **E**motion **A**ppearing **R**eal. The simulation is driven and energised by fear because Wetiko/Yaldabaoth (fear) *are* the simulation. Fear is the absence of love and Wetiko is the absence of love.

Wetiko today

We can now view current events from this level of perspective. The 'Covid' hoax has generated momentous amounts of ongoing fear, anxiety, depression and despair which have empowered Wetiko. No wonder people like Gates have been the instigators when they are Wetiko incarnate and exhibit every trait of Wetiko in the extreme. See how cold and unemotional these people are like Gates and his cronies, how dead of eye they are. That's Wetiko. Sabbatians are Wetiko and everything they control including the World Health Organization, Big Pharma and the 'vaccine' makers, national 'health'

hierarchies, corporate media, Silicon Valley, the banking system, and the United Nations with its planned transformation into world government. All are controlled and possessed by the Wetiko distortion into distorting human society in its image. We are with this knowledge at the gateway to understanding the world. Divisions of race, culture, creed and sexuality are diversions to hide the real division between those possessed and influenced by Wetiko and those that are not. The 'Covid' hoax has brought both clearly into view. Human behaviour is not about race. Tyrants and dictatorships come in all colours and creeds. What unites the US president bombing the innocent and an African tribe committing genocide against another as in Rwanda? What unites them? *Wetiko*. All wars are Wetiko, all genocide is Wetiko, all hunger over centuries in a world of plenty is Wetiko. Children going to bed hungry, including in the West, is Wetiko. Cult-generated Woke racial divisions that focus on the body are designed to obscure the reality that divisions in behaviour are manifestations of mind, not body. Obsession with body identity and group judgement is a means to divert attention from the real source of behaviour – mind and perception. Conflict sown by the Woke both within themselves and with their target groups are Wetiko providing lunch for itself through still more agents of the division, chaos, and fear on which it feeds. The Cult is seeking to assimilate the entirety of humanity and all children and young people into the Wetiko frequency by manipulating them into states of fear and despair. Witness all the suicide and psychological unravelling since the spring of 2020. Wetiko psychopaths want to impose a state of unquestioning obedience to authority which is no more than a conduit for Wetiko to enforce its will and assimilate humanity into itself. It needs us to believe that resistance is futile when it fears resistance and even more so the game-changing non-cooperation with its impositions. It can use violent resistance for its benefit. Violent impositions and violent resistance are *both* Wetiko. The Power of Love with its Power of No will sweep Wetiko from our world. Wetiko and its Cult know that. They just don't want us to know.

AI Wetiko

This brings me to AI or artificial intelligence and something else Wetikos don't want us to know. What is AI *really*? I know about computer code algorithms and AI that learns from data input. These, however, are more diversions, the expeditionary force, for the real AI that they want to connect to the human brain as promoted by Silicon Valley Wetikos like Kurzweil. What is this AI? It is the frequency of *Wetiko*, the frequency of the Archons. The connection of AI to the human brain is the connection of the Wetiko frequency to create a Wetiko hive mind and complete the job of assimilation. The hive mind is planned to be controlled from Israel and China which are both 100 percent owned by Wetiko Sabbatians. The assimilation process has been going on minute by minute in the 'smart' era which fused with the 'Covid' era. We are told that social media is scrambling the minds of the young and changing their personality. This is true, but what is social media? Look more deeply at how it works, how it creates divisions and conflict, the hostility and cruelty, the targeting of people until they are destroyed. That's Wetiko. Social media is manipulated to tune people to the Wetiko frequency with all the emotional exploitation tricks employed by platforms like Facebook and its Wetiko front man, Zuckerberg. Facebook's Instagram announced a new platform for children to overcome a legal bar on them using the main site. This is more Wetiko exploitation and manipulation of kids. Amnesty International likened the plan to foxes offering to guard the henhouse and said it was incompatible with human rights. Since when did Wetiko or Zuckerberg (I repeat myself) care about that? Would Brin and Page at Google, Wojcicki at YouTube, Bezos at Amazon and whoever the hell runs Twitter act as they do if they were not channelling Wetiko? Would those who are developing technologies for no other reason than human control? How about those designing and selling technologies to kill people and Big Pharma drug and 'vaccine' producers who know they will end or devastate lives? Quite a thought for these people to consider is that if you are Wetiko in a human life you are Wetiko on the 'other side' unless your frequency

changes and that can only change by a change of perception which becomes a change of behaviour. Where Gates is going does not bear thinking about although perhaps that's exactly where he wants to go. Either way, that's where he's going. His frequency will make it so.

The frequency lair

I have been saying for a long time that a big part of the addiction to smartphones and devices is that a frequency is coming off them that entraps the mind. People spend ages on their phones and sometimes even a minute or so after they put them down they pick them up again and it all repeats. 'Covid' lockdowns will have increased this addiction a million times for obvious reasons. Addictions to alcohol overindulgence and drugs are another way that Wetiko entraps consciousness to attach to its own. Both are symptoms of low-vibrational psychological distress which alcoholism and drug addiction further compound. Do we think it's really a coincidence that access to them is made so easy while potions that can take people into realms beyond the simulation are banned and illegal? I have explored smartphone addiction in other books, the scale is mind-blowing, and that level of addiction does not come without help. Tech companies that make these phones are Wetiko and they will have no qualms about destroying the minds of children. We are seeing again with these companies the Wetiko perceptual combination of psychopathic enforcers and weak and meek unquestioning compliance by the rank and file.

The global Smart Grid is the Wetiko Grid and it is crucial to complete the Cult endgame. The simulation is radiation and we are being deluged with technological radiation on a devastating scale. Wetiko frauds like Elon Musk serve Cult interests while occasionally criticising them to maintain his street-cred. 5G and other forms of Wi-Fi are being directed at the earth from space on a volume and scale that goes on increasing by the day. Elon Musk's (officially) SpaceX Starlink project is in the process of putting tens of thousands of satellites in low orbit to cover every inch of the planet with 5G and other Wi-Fi to create Kurzweil's global 'cloud' to which the

human mind is planned to be attached very soon. SpaceX has approval to operate 12,000 satellites with more than 1,300 launched at the time of writing and applications filed for *30,000* more. Other operators in the Wi-Fi, 5G, low-orbit satellite market include OneWeb (UK), Telesat (Canada), and AST & Science (US). Musk tells us that AI could be the end of humanity and then launches a company called Neuralink to connect the human brain to computers. Musk's (in theory) Tesla company is building electric cars and the driverless vehicles of the smart control grid. As frauds and bullshitters go Elon Musk in my opinion is Major League.

5G and technological radiation in general are destructive to human health, genetics and psychology and increasing the strength of artificial radiation underpins the five-sense perceptual bubbles which are themselves expressions of radiation or electromagnetism. Freedom activist John Whitehead was so right with his 'databit by databit, we are building our own electronic concentration camps'. The Smart Grid and 5G is a means to control the human mind and infuse perceptual information into The Field to influence anyone in sync with its frequency. You can change perception and behaviour en masse if you can manipulate the population into those levels of frequency and this is happening all around us today. The arrogance of Musk and his fellow Cult operatives knows no bounds in the way that we see with Gates. Musk's satellites are so many in number already they are changing the night sky when viewed from Earth. The astronomy community has complained about this and they have seen nothing yet. Some consequences of Musk's Wetiko hubris include: Radiation; visible pollution of the night sky; interference with astronomy and meteorology; ground and water pollution from intensive use of increasingly many spaceports; accumulating space debris; continual deorbiting and burning up of aging satellites, polluting the atmosphere with toxic dust and smoke; and ever-increasing likelihood of collisions. A collective public open letter of complaint to Musk said:

> We are writing to you … because SpaceX is in process of surrounding the Earth with a network of thousands of satellites whose very purpose is to irradiate every square inch of the

Earth. SpaceX, like everyone else, is treating the radiation as if it were not there. As if the mitochondria in our cells do not depend on electrons moving undisturbed from the food we digest to the oxygen we breathe.

As if our nervous systems and our hearts are not subject to radio frequency interference like any piece of electronic equipment. As if the cancer, diabetes, and heart disease that now afflict a majority of the Earth's population are not metabolic diseases that result from interference with our cellular machinery. As if insects everywhere, and the birds and animals that eat them, are not starving to death as a result.

People like Musk and Gates believe in their limitless Wetiko arrogance that they can do whatever they like to the world because they own it. Consequences for humanity are irrelevant. It's absolutely time that we stopped taking this shit from these self-styled masters of the Earth when you consider where this is going.

Why is the Cult so anti-human?

I hear this question often: Why would they do this when it will affect them, too? Ah, but will it? Who is this *them*? Forget their bodies. They are just vehicles for Wetiko consciousness. When you break it all down to the foundations we are looking at a state of severely distorted consciousness targeting another state of consciousness for assimilation. The rest is detail. The simulation is the fly-trap in which unique sensations of the five senses create a cycle of addiction called reincarnation. Renegade Minds see that everything which happens in our reality is a smaller version of the whole picture in line with the holographic principle. Addiction to the radiation of smart technology is a smaller version of addiction to the whole simulation. Connecting the body/brain to AI is taking that addiction on a giant step further to total ongoing control by assimilating human incarnate consciousness into Wetiko. I have watched during the 'Covid' hoax how many are becoming ever more profoundly attached to Wetiko's perceptual calling cards of aggressive response to any other point of view ('There is no other god but me'), psychopathic lack of compassion and empathy, and servile submission to the narrative and will of authority. Wetiko is the psychopaths *and* subservience to psychopaths. The Cult of Wetiko is

so anti-human because it is *not* human. It embarked on a mission to destroy human by targeting everything that it means to be human and to survive as human. 'Covid' is not the end, just a means to an end. The Cult with its Wetiko consciousness is seeking to change Earth systems, including the atmosphere, to suit them, not humans. The gathering bombardment of 5G alone from ground and space is dramatically changing The Field with which the five senses interact. There is so much more to come if we sit on our hands and hope it will all go away. It is not meant to go away. It is meant to get ever more extreme and we need to face that while we still can – just.

Carbon dioxide is the gas of life. Without that human is over. Kaput, gone, history. No natural world, no human. The Cult has created a cock and bull story about carbon dioxide and climate change to justify its reduction to the point where Gates and the ignoramus Biden 'climate chief' John Kerry want to suck it out of the atmosphere. Kerry wants to do this because his master Gates does. Wetikos have made the gas of life a demon with the usual support from the Wokers of Extinction Rebellion and similar organisations and the bewildered puppet-child that is Greta Thunberg who was put on the world stage by Klaus Schwab and the World Economic Forum. The name Extinction Rebellion is both ironic and as always Wetiko inversion. The gas that we need to survive must be reduced to save us from extinction. The most basic need of human is oxygen and we now have billions walking around in face nappies depriving body and brain of this essential requirement of human existence. More than that 5G at 60 gigahertz interacts with the oxygen molecule to reduce the amount of oxygen the body can absorb into the bloodstream. The obvious knock-on consequences of that for respiratory and cognitive problems and life itself need no further explanation. Psychopaths like Musk are assembling a global system of satellites to deluge the human atmosphere with this insanity. The man should be in jail. Here we have two most basic of human needs, oxygen and carbon dioxide, being dismantled.

Two others, water and food, are getting similar treatment with the United Nations Agendas 21 and 2030 – the Great Reset – planning to

centrally control all water and food supplies. People will not even own rain water that falls on their land. Food is affected at the most basic level by reducing carbon dioxide. We have genetic modification or GMO infiltrating the food chain on a mass scale, pesticides and herbicides polluting the air and destroying the soil. Freshwater fish that provide livelihoods for 60 million people and feed hundreds of millions worldwide are being 'pushed to the brink' according the conservationists while climate change is the only focus. Now we have Gates and Schwab wanting to dispense with current food sources all together and replace them with a synthetic version which the Wetiko Cult would control in terms of production and who eats and who doesn't. We have been on the Totalitarian Tiptoe to this for more than 60 years as food has become ever more processed and full of chemical shite to the point today when it's not natural food at all. As Dr Tom Cowan says: 'If it has a label don't eat it.' Bill Gates is now the biggest owner of farmland in the United States and he does nothing without an ulterior motive involving the Cult. Klaus Schwab wrote: 'To feed the world in the next 50 years we will need to produce as much food as was produced in the last 10,000 years … food security will only be achieved, however, if regulations on genetically modified foods are adapted to reflect the reality that gene editing offers a precise, efficient and safe method of improving crops.' Liar. People and the world are being targeted with aluminium through vaccines, chemtrails, food, drink cans, and endless other sources when aluminium has been linked to many health issues including dementia which is increasing year after year. Insects, bees and wildlife essential to the food chain are being deleted by pesticides, herbicides and radiation which 5G is dramatically increasing with 6G and 7G to come. The pollinating bee population is being devastated while wildlife including birds, dolphins and whales are having their natural radar blocked by the effects of ever-increasing radiation. In the summer windscreens used to be splattered with insects so numerous were they. It doesn't happen now. Where have they gone?

Synthetic everything

The Cult is introducing genetically-modified versions of trees, plants and insects including a Gates-funded project to unleash hundreds of millions of genetically-modified, lab-altered and patented male mosquitoes to mate with wild mosquitoes and induce genetic flaws that cause them to die out. Clinically-insane Gates-funded Japanese researchers have developed mosquitos that spread vaccine and are dubbed 'flying vaccinators'. Gates is funding the modification of weather patterns in part to sell the myth that this is caused by carbon dioxide and he's funding geoengineering of the skies to change the atmosphere. Some of this came to light with the Gates-backed plan to release tonnes of chalk into the atmosphere to 'deflect the Sun and cool the planet'. Funny how they do this while the heating effect of the Sun is not factored into climate projections focussed on carbon dioxide. The reason is that they want to reduce carbon dioxide (so don't mention the Sun), but at the same time they do want to reduce the impact of the Sun which is so essential to human life and health. I have mentioned the sun-cholesterol-vitamin D connection as they demonise the Sun with warnings about skin cancer (caused by the chemicals in sun cream they tell you to splash on). They come from the other end of the process with statin drugs to reduce cholesterol that turns sunlight into vitamin D. A lack of vitamin D leads to a long list of health effects and how vitamin D levels must have fallen with people confined to their homes over 'Covid'. Gates is funding other forms of geoengineering and most importantly chemtrails which are dropping heavy metals, aluminium and self-replicating nanotechnology onto the Earth which is killing the natural world. See *Everything You Need To Know, But Have Never Been Told* for the detailed background to this.

Every human system is being targeted for deletion by a force that's not human. The Wetiko Cult has embarked on the process of transforming the human body from biological to synthetic biological as I have explained. Biological is being replaced by the artificial and synthetic – Archontic 'countermimicry' – right across human society. The plan eventually is to dispense with the human body altogether

and absorb human consciousness – which it wouldn't really be by then – into cyberspace (the simulation which is Wetiko/Yaldabaoth). Preparations for that are already happening if people would care to look. The alternative media rightly warns about globalism and 'the globalists', but this is far bigger than that and represents the end of the human race as we know it. The 'bad copy' of prime reality that Gnostics describe was a bad copy of harmony, wonder and beauty to start with before Wetiko/Yaldabaoth set out to change the simulated 'copy' into something very different. The process was slow to start with. Entrapped humans in the simulation timeline were not technologically aware and they had to be brought up to intellectual speed while being suppressed spiritually to the point where they could build their own prison while having no idea they were doing so. We have now reached that stage where technological intellect has the potential to destroy us and that's why events are moving so fast. Central American shaman Don Juan Matus said:

> Think for a moment, and tell me how you would explain the contradictions between the intelligence of man the engineer and the stupidity of his systems of belief, or the stupidity of his contradictory behaviour. Sorcerers believe that the predators have given us our systems of beliefs, our ideas of good and evil; our social mores. They are the ones who set up our dreams of success or failure. They have given us covetousness, greed, and cowardice. It is the predator who makes us complacent, routinary, and egomaniacal.
>
> In order to keep us obedient and meek and weak, the predators engaged themselves in a stupendous manoeuvre – stupendous, of course, from the point of view of a fighting strategist; a horrendous manoeuvre from the point of those who suffer it. They gave us their mind. The predators' mind is baroque, contradictory, morose, filled with the fear of being discovered any minute now.

For 'predators' see Wetiko, Archons, Yaldabaoth, Jinn, and all the other versions of the same phenomenon in cultures and religions all over the world. The theme is always the same because it's true and it's real. We have reached the point where we have to deal with it. The question is – how?

Don't fight – walk away

I thought I'd use a controversial subheading to get things moving in terms of our response to global fascism. What do you mean 'don't fight'? What do you mean 'walk away'? We've got to fight. We can't walk away. Well, it depends what we mean by fight and walk away. If fighting means physical combat we are playing Wetiko's game and falling for its trap. It wants us to get angry, aggressive, and direct hate and hostility at the enemy we think we must fight. Every war, every battle, every conflict, has been fought with Wetiko leading both sides. It's what it does. Wetiko wants a fight, anywhere, any place. Just hit me, son, so I can hit you back. Wetiko hits Wetiko and Wetiko hits Wetiko in return. I am very forthright as you can see in exposing Wetikos of the Cult, but I don't hate them. I refuse to hate them. It's what they want. What you hate you become. What you *fight* you become. Wokers, 'anti-haters' and 'anti-fascists' prove this every time they reach for their keyboards or don their balaclavas. By walk away I mean to disengage from Wetiko which includes ceasing to cooperate with its tyranny. Paul Levy says of Wetiko:

> The way to 'defeat' evil is not to try to destroy it (for then, in playing evil's game, we have already lost), but rather, to find the invulnerable place within ourselves where evil is unable to vanquish us – this is to truly 'win' our battle with evil.

Wetiko is everywhere in human society and it's been on steroids since the 'Covid' hoax. Every shouting match over wearing masks has Wetiko wearing a mask and Wetiko not wearing one. It's an electrical circuit of push and resist, push and resist, with Wetiko pushing *and* resisting. Each polarity is Wetiko empowering itself. Dictionary definitions of 'resist' include 'opposing, refusing to accept or comply with' and the word to focus on is 'opposing'. What form does this take – setting police cars alight or 'refusing to accept or comply with'? The former is Wetiko opposing Wetiko while the other points the way forward. This is the difference between those aggressively demanding that government fascism must be obeyed who stand in stark contrast to the great majority of Pushbackers. We saw this clearly with a march by thousands of Pushbackers against lockdown in London followed days later by a Woker-hijacked

protest in Bristol in which police cars were set on fire. Masks were virtually absent in London and widespread in Bristol. Wetiko wants lockdown on every level of society and infuses its aggression to police it through its unknowing stooges. Lockdown protesters are the ones with the smiling faces and the hugs, The two blatantly obvious states of being – getting more obvious by the day – are the result of Wokers and their like becoming ever more influenced by the simulation Field of Wetiko and Pushbackers ever more influenced by The Field of a far higher vibration beyond the simulation. Wetiko can't invade the heart which is where most lockdown opponents are coming from. It's the heart that allows them to see through the lies to the truth in ways I will be highlighting.

Renegade Minds know that calmness is the place from which wisdom comes. You won't find wisdom in a hissing fit and wisdom is what we need in abundance right now. Calmness is not weakness – you don't have to scream at the top of your voice to be strong. Calmness is indeed a sign of strength. 'No' means I'm not doing it. *NOOOO!!!* doesn't mean you're not doing it even more. Volume does not advance 'No – I'm not doing it'. You are just not doing it. Wetiko possessed and influenced don't know how to deal with that. Wetiko wants a fight and we should not give it one. What it needs more than anything is our *cooperation* and we should not give that either. Mass rallies and marches are great in that they are a visual representation of feeling, but if it ends there they are irrelevant. You demand that Wetikos act differently? Well, they're not going to are they? They are Wetikos. We don't need to waste our time demanding that something doesn't happen when that will make no difference. We need to delete the means that *allows* it to happen. This, invariably, is our cooperation. You can demand a child stop firing a peashooter at the dog or you can refuse to buy the peashooter. If you provide the means you are cooperating with the dog being smacked on the nose with a pea. How can the authorities enforce mask-wearing if millions in a country refuse? What if the 74 million Pushbackers that voted for Trump in 2020 refused to wear masks, close their businesses or stay in their homes. It would be unenforceable. The

few control the many through the compliance of the many and that's always been the dynamic be it 'Covid' regulations or the Roman Empire. I know people can find it intimidating to say no to authority or stand out in a crowd for being the only one with a face on display; but it has to be done or it's over. I hope I've made clear in this book that where this is going will be far more intimidating than standing up now and saying 'No' – I will not cooperate with my own enslavement and that of my children. There might be consequences for some initially, although not so if enough do the same. The question that must be addressed is what is going to happen if we don't? It is time to be strong and unyieldingly so. No means no. Not here and there, but *everywhere* and *always*. I have refused to wear a mask and obey all the other nonsense. I will not comply with tyranny. I repeat: Fascism is not imposed by fascists – there are never enough of them. Fascism is imposed by the population acquiescing to fascism. *I will not do it*. I will die first, or my body will. Living meekly under fascism is a form of death anyway, the death of the spirit that Martin Luther King described.

Making things happen

We must not despair. This is not over till it's over and it's far from that. The 'fat lady' must refuse to sing. The longer the 'Covid' hoax has dragged on and impacted on more lives we have seen an awakening of phenomenal numbers of people worldwide to the realisation that what they have believed all their lives is not how the world really is. Research published by the system-serving University of Bristol and King's College London in February, 2021, concluded: 'One in every 11 people in Britain say they trust David Icke's take on the coronavirus pandemic.' It will be more by now and we have gathering numbers to build on. We must urgently progress from seeing the scam to ceasing to cooperate with it. Prominent German lawyer Reiner Fuellmich, also licenced to practice law in America, is doing a magnificent job taking the legal route to bring the psychopaths to justice through a second Nuremberg tribunal for crimes against humanity. Fuellmich has an impressive record of

beating the elite in court and he formed the German Corona Investigative Committee to pursue civil charges against the main perpetrators with a view to triggering criminal charges. Most importantly he has grasped the foundation of the hoax – the PCR test not testing for the 'virus' – and Christian Drosten is therefore on his charge sheet along with Gates frontman Tedros at the World Health Organization. Major players must be not be allowed to inflict their horrors on the human race without being brought to book. A life sentence must follow for Bill Gates and the rest of them. A group of researchers has also indicted the government of Norway for crimes against humanity with copies sent to the police and the International Criminal Court. The lawsuit cites participation in an internationally-planned false pandemic and violation of international law and human rights, the European Commission's definition of human rights by coercive rules, Nuremberg and Hague rules on fundamental human rights, and the Norwegian constitution. We must take the initiative from hereon and not just complain, protest and react.

There are practical ways to support vital mass non-cooperation. Organising in numbers is one. Lockdown marches in London in the spring in 2021 were mass non-cooperation that the authorities could not stop. There were too many people. Hundreds of thousands walked the London streets in the centre of the road for mile after mile while the Face-Nappies could only look on. They were determined, but calm, and just *did it* with no histrionics and lots of smiles. The police were impotent. Others are organising group shopping without masks for mutual support and imagine if that was happening all over. Policing it would be impossible. If the store refuses to serve people in these circumstances they would be faced with a long line of trolleys full of goods standing on their own and everything would have to be returned to the shelves. How would they cope with that if it kept happening? I am talking here about moving on from complaining to being pro-active; from watching things happen to making things happen. I include in this our relationship with the police. The behaviour of many Face-Nappies

has been disgraceful and anyone who thinks they would never find concentration camp guards in the 'enlightened' modern era have had that myth busted big-time. The period and setting may change – Wetikos never do. I watched film footage from a London march in which a police thug viciously kicked a protestor on the floor who had done nothing. His fellow Face-Nappies stood in a ring protecting him. What he did was a criminal assault and with a crowd far outnumbering the police this can no longer be allowed to happen unchallenged. I get it when people chant 'shame on you' in these circumstances, but that is no longer enough. They *have* no shame those who do this. Crowds needs to start making a citizen's arrest of the police who commit criminal offences and brutally attack innocent people and defenceless women. A citizen's arrest can be made under section 24A of the UK Police and Criminal Evidence (PACE) Act of 1984 and you will find something similar in other countries. I prefer to call it a Common Law arrest rather than citizen's for reasons I will come to shortly. Anyone can arrest a person committing an indictable offence or if they have reasonable grounds to suspect they are committing an indictable offence. On both counts the attack by the police thug would have fallen into this category. A citizen's arrest can be made to stop someone:

- Causing physical injury to himself or any other person
- Suffering physical injury
- Causing loss of or damage to property
- Making off before a constable can assume responsibility for him

A citizen's arrest may also be made to prevent a breach of the peace under Common Law and if they believe a breach of the peace will happen or anything related to harm likely to be done or already done in their presence. This is the way to go I think – the Common Law version. If police know that the crowd and members of the public will no longer be standing and watching while they commit

their thuggery and crimes they will think twice about acting like Brownshirts and Blackshirts.

Common Law – common sense

Mention of Common Law is very important. Most people think the law is the law as in one law. This is not the case. There are two bodies of law, Common Law and Statute Law, and they are not the same. Common Law is founded on the simple premise of do no harm. It does not recognise victimless crimes in which no harm is done while Statute Law does. There is a Statute Law against almost everything. So what is Statute Law? Amazingly it's the law of the *sea* that was brought ashore by the Cult to override the law of the land which is Common Law. They had no right to do this and as always they did it anyway. They had to. They could not impose their will on the people through Common Law which only applies to do no harm. How could you stitch up the fine detail of people's lives with that? Instead they took the law of the sea, or Admiralty Law, and applied it to the population. Statute Law refers to all the laws spewing out of governments and their agencies including all the fascist laws and regulations relating to 'Covid'. The key point to make is that Statute Law is *contract law*. It only applies between *contracting* corporations. Most police officers don't even know this. They have to be kept in the dark, too. Long ago when merchants and their sailing ships began to trade with different countries a contractual law was developed called Admiralty Law and other names. Again it only applied to *contracts* agreed between *corporate* entities. If there is no agreed contract the law of the sea had no jurisdiction *and that still applies to its new alias of Statute Law*. The problem for the Cult when the law of the sea was brought ashore was an obvious one. People were not corporations and neither were government entities. To overcome the latter they made governments and all associated organisations corporations. All the institutions are *private corporations* and I mean governments and their agencies, local councils, police, courts, military, US states, the whole lot. Go to the

Dun and Bradstreet corporate listings website for confirmation that they are all corporations. You are arrested by a private corporation called the police by someone who is really a private security guard and they take you to court which is another private corporation. Neither have jurisdiction over you unless you consent and *contract* with them. This is why you hear the mantra about law enforcement policing by *consent* of the people. In truth the people 'consent' only in theory through monumental trickery.

Okay, the Cult overcame the corporate law problem by making governments and institutions corporate entities; but what about people? They are not corporations are they? Ah ... well in a sense, and *only* a sense, they are. Not people exactly – the illusion of people. The Cult creates a corporation in the name of everyone at the time that their birth certificate is issued. Note birth/ *berth* certificate and when you go to court under the law of the sea on land you stand in a *dock*. These are throwbacks to the origin. My Common Law name is David Vaughan Icke. The name of the corporation created by the government when I was born is called Mr David Vaughan Icke usually written in capitals as MR DAVID VAUGHAN ICKE. That is not me, the living, breathing man. It is a fictitious corporate entity. The trick is to make you think that David Vaughan Icke and MR DAVID VAUGHAN ICKE are the same thing. *They are not*. When police charge you and take you to court they are prosecuting the corporate entity and not the living, breathing, man or woman. They have to trick you into identifying as the corporate entity and contracting with them. Otherwise they have no jurisdiction. They do this through a language known as legalese. Lawful and legal are not the same either. Lawful relates to Common Law and legal relates to Statute Law. Legalese is the language of Statue Law which uses terms that mean one thing to the public and another in legalese. Notice that when a police officer tells someone why they are being charged he or she will say at the end: 'Do you understand?' To the public that means 'Do you comprehend?' In legalese it means 'Do you stand under me?' Do you stand under my authority? If you say

yes to the question you are unknowingly agreeing to give them jurisdiction over you in a contract between two corporate entities.

This is a confidence trick in every way. Contracts have to be agreed between informed parties and if you don't know that David Vaughan Icke is agreeing to be the corporation MR DAVID VAUGHAN ICKE you cannot knowingly agree to contract. They are deceiving you and another way they do this is to ask for proof of identity. You usually show them a driving licence or other document on which your corporate name is written. In doing so you are accepting that you are that corporate entity when you are not. Referring to yourself as a 'person' or 'citizen' is also identifying with your corporate fiction which is why I made the Common Law point about the citizen's arrest. If you are approached by a police officer you identify yourself immediately as a living, breathing, man or woman and say 'I do not consent, I do not contract with you and I do not understand' or stand under their authority. I have a Common Law birth certificate as a living man and these are available at no charge from commonlawcourt.com. Businesses registered under the Statute Law system means that its laws apply. There are, however, ways to run a business under Common Law. Remember all 'Covid' laws and regulations are Statute Law – the law of *contracts* and you do not have to contract. This doesn't mean that you can kill someone and get away with it. Common Law says do no harm and that applies to physical harm, financial harm etc. Police are employees of private corporations and there needs to be a new system of non-corporate Common Law constables operating outside the Statute Law system. If you go to davidicke.com and put Common Law into the search engine you will find videos that explain Common Law in much greater detail. It is definitely a road we should walk.

With all my heart

I have heard people say that we are in a spiritual war. I don't like the term 'war' with its Wetiko dynamic, but I know what they mean. Sweep aside all the bodily forms and we are in a situation in which two states of consciousness are seeking very different realities.

Wetiko wants upheaval, chaos, fear, suffering, conflict and control. The other wants love, peace, harmony, fairness and freedom. That's where we are. We should not fall for the idea that Wetiko is all-powerful and there's nothing we can do. Wetiko is not all-powerful. It's a joke, pathetic. It doesn't have to be, but it has made that choice for now. A handful of times over the years when I have felt the presence of its frequency I have allowed it to attach briefly so I could consciously observe its nature. The experience is not pleasant, the energy is heavy and dark, but the ease with which you can kick it back out the door shows that its real power is in persuading us that it has power. It's all a con. Wetiko is a con. It's a trickster and not a power that can control us if we unleash our own. The con is founded on manipulating humanity to give its power to Wetiko which recycles it back to present the illusion that it has power when its power is *ours* that we gave away. This happens on an energetic level and plays out in the world of the seen as humanity giving its power to Wetiko authority which uses that power to control the population when the power is only the power the population has handed over. How could it be any other way for billions to be controlled by a relative few? I have had experiences with people possessed by Wetiko and again you can kick its arse if you do it with an open heart. Oh yes – the *heart* which can transform the world of perceived 'matter'.

We are receiver-transmitters and processors of information, but what information and where from? Information is processed into perception in three main areas – the brain, the heart and the belly. These relate to thinking, knowing, and emotion. Wetiko wants us to be head and belly people which means we think within the confines of the Matrix simulation and low-vibrational emotional reaction scrambles balance and perception. A few minutes on social media and you see how emotion is the dominant force. Woke is all emotion and is therefore thought-free and fact-free. Our heart is something different. It *knows* while the head *thinks* and has to try to work it out because it doesn't know. The human energy field has seven prime vortexes which connect us with wider reality (Fig 23). Chakra means

'wheels of light' in the Sanskrit language of ancient India. The main ones are: The crown chakra on top of the head; brow (or 'third eye') chakra in the centre of the forehead; throat chakra; heart chakra in the centre of the chest; solar plexus chakra below the sternum; sacral chakra beneath the navel; and base chakra at the bottom of the spine. Each one has a particular function or functions. We feel anxiety and nervousness in the belly where the sacral chakra is located and this processes emotion that can affect the colon to give people 'the shits' or make them 'shit scared' when they are nervous. Chakras all play an important role, but the Mr and Mrs Big is the heart chakra which sits at the centre of the seven, above the chakras that connect us to the 'physical' and below those that connect with higher realms (or at least should). Here in the heart chakra we feel love, empathy and compassion – 'My heart goes out to you'. Those with closed hearts become literally 'heart-less' in their attitudes and behaviour (see Bill Gates). Native Americans portrayed Wetiko with what Paul Levy calls a 'frigid, icy heart, devoid of mercy' (see Bill Gates).

Figure 23: The chakra system which interpenetrates the human energy field. The heart chakra is the governor – or should be.

Wetiko trembles at the thought of heart energy which it cannot infiltrate. The frequency is too high. What it seeks to do instead is close the heart chakra vortex to block its perceptual and energetic influence. Psychopaths have 'hearts of stone' and emotionally-damaged people have 'heartache' and 'broken hearts'. The astonishing amount of heart disease is related to heart chakra

disruption with its fundamental connection to the 'physical' heart. Dr Tom Cowan has written an outstanding book challenging the belief that the heart is a pump and making the connection between the 'physical' and spiritual heart. Rudolph Steiner who was way ahead of his time said the same about the fallacy that the heart is a pump. *What*? The heart is not a pump? That's crazy, right? Everybody knows that. Read Cowan's *Human Heart, Cosmic Heart* and you will realise that the very idea of the heart as a pump is ridiculous when you see the evidence. How does blood in the feet so far from the heart get pumped horizontally up the body by the heart?? Cowan explains in the book the real reason why blood moves as it does. Our 'physical' heart is used to symbolise love when the source is really the heart vortex or spiritual heart which is our most powerful energetic connection to 'out there' expanded consciousness. That's why we feel *knowing* – intuitive knowing – in the centre of the chest. Knowing doesn't come from a process of thoughts leading to a conclusion. It is there in an instant all in one go. Our heart knows because of its connection to levels of awareness that *do* know. This is the meaning and source of intuition – intuitive *knowing*.

For the last more than 30 years of uncovering the global game and the nature of reality my heart has been my constant antenna for truth and accuracy. An American intelligence insider once said that I had quoted a disinformer in one of my books and yet I had only quoted the part that was true. He asked: 'How do you do that?' By using my heart antenna was the answer and anyone can do it. Heart-centred is how we are meant to be. With a closed heart chakra we withdraw into a closed mind and the bubble of five-sense reality. If you take a moment to focus your attention on the centre of your chest, picture a spinning wheel of light and see it opening and expanding. You will feel it happening, too, and perceptions of the heart like joy and love as the heart impacts on the mind as they interact. The more the chakra opens the more you will feel expressions of heart consciousness and as the process continues, and becomes part of you, insights and knowings will follow. An open

heart is connected to that level of awareness that knows all is *One*. You will see from its perspective that the fault-lines that divide us are only illusions to control us. An open heart does not process the illusions of race, creed and sexuality except as brief experiences for a consciousness that is all. Our heart does not see division, only unity (Figs 24 and 25). There's something else, too. Our hearts love to laugh. Mark Twain's quote that says 'The human race has one really effective weapon, and that is laughter' is really a reference to the heart which loves to laugh with the joy of knowing the true nature of infinite reality and that all the madness of human society is an illusion of the mind. Twain also said: 'Against the assault of laughter nothing can stand.' This is so true of Wetiko and the Cult. Their insecurity demands that they be taken seriously and their power and authority acknowledged and feared. We should do nothing of the sort. We should not get aggressive or fearful which their insecurity so desires. We should laugh in their face. Even in their no-face as police come over in their face-nappies and expect to be taken seriously. They don't take themselves seriously looking like that so why should we? Laugh in the face of intimidation. Laugh in the face of tyranny. You will see by its reaction that you have pressed all of its buttons. Wetiko does not know what to do in the face of laughter or when its targets refuse to concede their joy to fear. We have seen many examples during the 'Covid' hoax when people have expressed their energetic power and the string puppets of Wetiko retreat with their tail limp between their knees. Laugh – the world is bloody mad after all and if it's a choice between laughter and tears I know which way I'm going.

Figure 24: Head consciousness without the heart sees division and everything apart from everything else.

Figure 25: Heart consciousness sees everything as One.

'Vaccines' and the soul

The foundation of Wetiko/Archon control of humans is the separation of incarnate five-sense mind from the infinite 'I' and closing the heart chakra where the True 'I' lives during a human life. The goal has been to achieve complete separation in both cases. I was interested therefore to read an account by a French energetic healer of what she said she experienced with a patient who had been given the 'Covid' vaccine. Genuine energy healers can sense information and consciousness fields at different levels of being which are referred to as 'subtle bodies'. She described treating the patient who later returned after having, without the healer's knowledge, two doses of the 'Covid vaccine'. The healer said:

I noticed immediately the change, very heavy energy emanating from [the] subtle bodies. The scariest thing was when I was working on the heart chakra, I connected with her soul: it was detached from the physical body, it had no contact and it was, as if it was floating in a state of total confusion: a damage to the consciousness that loses contact with the physical body, i.e. with our biological machine, there is no longer any communication between them.

I continued the treatment by sending light to the heart chakra, the soul of the person, but it seemed that the soul could no longer receive any light, frequency or energy. It was a very powerful experience for me. Then I understood that this substance is indeed used to detach consciousness so that this consciousness can no longer interact through this body that it possesses in life, where there is no longer any contact, no frequency, no light, no more energetic balance or mind.

This would create a human that is rudderless and at the extreme almost zombie-like operating with a fractional state of consciousness at the mercy of Wetiko. I was especially intrigued by what the healer said in the light of the prediction by the highly-informed Rudolf Steiner more than a hundred years ago. He said:

In the future, we will eliminate the soul with medicine. Under the pretext of a 'healthy point of view', there will be a vaccine by which the human body will be treated as soon as possible directly at birth, so that the human being cannot develop the thought of the existence of soul and Spirit. To materialistic doctors will be entrusted the task of removing the soul of humanity.

As today, people are vaccinated against this disease or that disease, so in the future, children will be vaccinated with a substance that can be produced precisely in such a way that people, thanks to this vaccination, will be immune to being subjected to the 'madness' of spiritual life. He would be extremely smart, but he would not develop a conscience, and that is the true goal of some materialistic circles.

Steiner said the vaccine would detach the physical body from the etheric body (subtle bodies) and 'once the etheric body is detached the relationship between the universe and the etheric body would become extremely unstable, and man would become an automaton'. He said 'the physical body of man must be polished on this Earth by spiritual will – so the vaccine becomes a kind of arymanique (Wetiko) force' and 'man can no longer get rid of a given materialistic feeling'. Humans would then, he said, become 'materialistic of constitution and can no longer rise to the spiritual'. I have been writing for years about DNA being a receiver-transmitter of information that connects us to other levels of reality and these 'vaccines' changing DNA can be likened to changing an antenna and what it can transmit and receive. Such a disconnection would clearly lead to changes in personality and perception. Steiner further predicted the arrival of AI. Big Pharma 'Covid vaccine' makers, expressions of Wetiko, are testing their DNA-manipulating evil on children as I write with a view to giving the 'vaccine' to babies. If it's a soul-body disconnector – and I say that it is or can be – every child would be disconnected from 'soul' at birth and the 'vaccine' would create a closed system in which spiritual guidance from the greater self would play no part. This has been the ambition of Wetiko all

along. A Pentagon video from 2005 was leaked of a presentation explaining the development of vaccines to change behaviour by their effect on the brain. Those that believe this is not happening with the 'Covid' genetically-modifying procedure masquerading as a 'vaccine' should make an urgent appointment with Naivety Anonymous. Klaus Schwab wrote in 2018:

> Neurotechnologies enable us to better influence consciousness and thought and to understand many activities of the brain. They include decoding what we are thinking in fine levels of detail through new chemicals and interventions that can influence our brains to correct for errors or enhance functionality.

The plan is clear and only the heart can stop it. With every heart that opens, every mind that awakens, Wetiko is weakened. Heart and love are far more powerful than head and hate and so nothing like a majority is needed to turn this around.

Beyond the Phantom

Our heart is the prime target of Wetiko and so it must be the answer to Wetiko. We *are* our heart which is part of one heart, the infinite heart. Our heart is where the true self lives in a human life behind firewalls of five-sense illusion when an imposter takes its place – *Phantom Self*; but our heart waits patiently to be set free any time we choose to see beyond the Phantom, beyond Wetiko. A Wetikoed Phantom Self can wreak mass death and destruction while the love of forever is locked away in its heart. The time is here to unleash its power and let it sweep away the fear and despair that is Wetiko. Heart consciousness does not seek manipulated, censored, advantage for its belief or religion, its activism and desires. As an expression of the One it treats all as One with the same rights to freedom and opinion. Our heart demands fairness for itself no more than for others. From this unity of heart we can come together in mutual support and transform this Wetikoed world into what reality is meant to be – a place of love, joy, happiness, fairness, justice and freedom. Wetiko has another agenda and that's why the world is as

it is, but enough of this nonsense. Wetiko can't stay where hearts are open and it works so hard to keep them closed. Fear is its currency and its food source and love in its true sense has no fear. Why would love have fear when it knows it is *All That Is, Has Been, And Ever Can Be* on an eternal exploration of all possibility? Love in this true sense is not the physical attraction that passes for love. This can be an expression of it, yes, but Infinite Love, a love without condition, goes far deeper to the core of all being. It *is* the core of all being. Infinite realty was born from love beyond the illusions of the simulation. Love infinitely expressed is the knowing that all is One and the swiftly-passing experience of separation is a temporary hallucination. You cannot disconnect from Oneness; you can only *perceive* that you have and withdraw from its influence. This is the most important of all perception trickery by the mind parasite that is Wetiko and the foundation of all its potential for manipulation.

If we open our hearts, open the sluice gates of the mind, and redefine self-identity amazing things start to happen. Consciousness expands or contracts in accordance with self-identity. When true self is recognised as infinite awareness and label self – Phantom Self – is seen as only a series of brief experiences life is transformed. Consciousness expands to the extent that self-identity expands and everything changes. You see unity, not division, the picture, not the pixels. From this we can play the long game. No more is an experience something in and of itself, but a fleeting moment in the eternity of forever. Suddenly people in uniform and dark suits are no longer intimidating. Doing what your heart knows to be right is no longer intimidating and consequences for those actions take on the same nature of a brief experience that passes in the blink of an infinite eye. Intimidation is all in the mind. Beyond the mind there is no intimidation.

An open heart does not consider consequences for what it knows to be right. To do so would be to consider not doing what it knows to be right and for a heart in its power that is never an option. The Renegade Mind is really the Renegade Heart. Consideration of consequences will always provide a getaway car for the mind and

the heart doesn't want one. What is right in the light of what we face today is to stop cooperating with Wetiko in all its forms and to do it without fear or compromise. You cannot compromise with tyranny when tyranny always demands more until it has everything. Life is your perception and you are your destiny. Change your perception and you change your life. Change collective perception and we change the world.

Come on people … One human family, One heart, One goal … *FREEEEEEDOM*!

We must settle for nothing less.

Postscript

The big scare story as the book goes to press is the 'Indian' variant and the world is being deluged with propaganda about the 'Covid catastrophe' in India which mirrors in its lies and misrepresentations what happened in Italy before the first lockdown in 2020.

The *New York Post* published a picture of someone who had 'collapsed in the street from Covid' in India in April, 2021, which was actually taken during a gas leak in May, 2020. Same old, same old. Media articles in mid-February were asking why India had been so untouched by 'Covid' and then as their vaccine rollout gathered pace the alleged 'cases' began to rapidly increase. Indian 'Covid vaccine' maker Bharat Biotech was funded into existence by the Bill and Melinda Gates Foundation (the pair announced their divorce in May, 2021, which is a pity because they so deserve each other). The Indian 'Covid crisis' was ramped up by the media to terrify the world and prepare people for submission to still more restrictions. The scam that worked the first time was being repeated only with far more people seeing through the deceit. Davidicke.com and Ickonic.com have sought to tell the true story of what is happening by talking to people living through the Indian nightmare which has nothing to do with 'Covid'. We posted a letter from 'Alisha' in Pune who told a very different story to government and media mendacity. She said scenes of dying people and overwhelmed hospitals were designed to hide what was really happening – genocide and starvation. Alisha said that millions had already died of starvation during the ongoing lockdowns while government and media were lying and making it look like the 'virus':

Restaurants, shops, gyms, theatres, basically everything is shut. The cities are ghost towns. Even so-called 'essential' businesses are only open till 11am in the morning. You basically have just an hour to buy food and then your time is up.

Inter-state travel and even inter-district travel is banned. The cops wait at all major crossroads to question why you are traveling outdoors or to fine you if you are not wearing a mask.

The medical community here is also complicit in genocide, lying about hospitals being full and turning away people with genuine illnesses, who need immediate care. They have even created a shortage of oxygen cylinders.

This is the classic Cult modus operandi played out in every country. Alisha said that people who would not have a PCR test not testing for the 'virus' were being denied hospital treatment. She said the people hit hardest were migrant workers and those in rural areas. Most businesses employed migrant workers and with everything closed there were no jobs, no income and no food. As a result millions were dying of starvation or malnutrition. All this was happening under Prime Minister Narendra Modi, a 100-percent asset of the Cult, and it emphasises yet again the scale of pure anti-human evil we are dealing with. Australia banned its people from returning home from India with penalties for trying to do so of up to five years in jail and a fine of £37,000. The manufactured 'Covid' crisis in India was being prepared to justify further fascism in the West. Obvious connections could be seen between the Indian 'vaccine' programme and increased 'cases' and this became a common theme. The Seychelles, the most per capita 'Covid vaccinated' population in the world, went back into lockdown after a 'surge of cases'.

Long ago the truly evil Monsanto agricultural biotechnology corporation with its big connections to Bill Gates devastated Indian farming with genetically-modified crops. Human rights activist Gurcharan Singh highlighted the efforts by the Indian government to complete the job by destroying the food supply to hundreds of millions with 'Covid' lockdowns. He said that 415 million people at the bottom of the disgusting caste system (still going whatever they say) were below the poverty line and struggled to feed themselves every year. Now the government was imposing lockdown at just the

time to destroy the harvest. This deliberate policy was leading to mass starvation. People may reel back at the suggestion that a government would do that, but Wetiko-controlled 'leaders' are capable of any level of evil. In fact what is described in India is in the process of being instigated worldwide. The food chain and food supply are being targeted at every level to cause world hunger and thus control. Bill Gates is not the biggest owner of farmland in America for no reason and destroying access to food aids both the depopulation agenda and the plan for synthetic 'food' already being funded into existence by Gates. Add to this the coming hyper-inflation from the suicidal creation of fake 'money' in response to 'Covid' and the breakdown of container shipping systems and you have a cocktail that can only lead one way and is meant to. The Cult plan is to crash the entire system to 'build back better' with the Great Reset.

'Vaccine' transmission

Reports from all over the world continue to emerge of women suffering menstrual and fertility problems after having the fake 'vaccine' and of the non-'vaccinated' having similar problems when interacting with the 'vaccinated'. There are far too many for 'coincidence' to be credible. We've had menopausal women getting periods, others having periods stop or not stopping for weeks, passing clots, sometimes the lining of the uterus, breast irregularities, and miscarriages (which increased by 400 percent in parts of the United States). Non-'vaccinated' men and children have suffered blood clots and nose bleeding after interaction with the 'vaccinated'. Babies have died from the effects of breast milk from a 'vaccinated' mother. Awake doctors – the small minority – speculated on the cause of non-'vaccinated' suffering the same effects as the 'vaccinated'. Was it nanotechnology in the synthetic substance transmitting frequencies or was it a straight chemical bioweapon that was being transmitted between people? I am not saying that some kind of chemical transmission is not one possible answer, but the foundation of all that the Cult does is frequency and

this is fertile ground for understanding how transmission can happen. American doctor Carrie Madej, an internal medicine physician and osteopath, has been practicing for the last 20 years, teaching medical students, and she says attending different meetings where the agenda for humanity was discussed. Madej, who operates out of Georgia, did not dismiss other possible forms of transmission, but she focused on frequency in search of an explanation for transmission. She said the Moderna and Pfizer 'vaccines' contained nano-lipid particles as a key component. This was a brand new technology never before used on humanity. 'They're using a nanotechnology which is pretty much little tiny computer bits … nanobots or hydrogel.' Inside the 'vaccines' was 'this sci-fi kind of substance' which suppressed immune checkpoints to get into the cell. I referred to this earlier as the 'Trojan horse' technique that tricks the cell into opening a gateway for the self-replicating synthetic material and while the immune system is artificially suppressed the body has no defences. Madej said the substance served many purposes including an on-demand ability to 'deliver the payload' and using the nano 'computer bits' as biosensors in the body. 'It actually has the ability to accumulate data from your body, like your breathing, your respiration, thoughts, emotions, all kinds of things.'

She said the technology obviously has the ability to operate through Wi-Fi and transmit and receive energy, messages, frequencies or impulses. 'Just imagine you're getting this new substance in you and it can react to things all around you, the 5G, your smart device, your phones.' We had something completely foreign in the human body that had never been launched large scale at a time when we were seeing 5G going into schools and hospitals (plus the Musk satellites) and she believed the 'vaccine' transmission had something to do with this: '… if these people have this inside of them … it can act like an antenna and actually transmit it outwardly as well.' The synthetic substance produced its own voltage and so it could have that kind of effect. This fits with my own contention that the nano receiver-transmitters are designed to connect people to the

Smart Grid and break the receiver-transmitter connection to expanded consciousness. That would explain the French energy healer's experience of the disconnection of body from 'soul' with those who have had the 'vaccine'. The nanobots, self-replicating inside the body, would also transmit the synthetic frequency which could be picked up through close interaction by those who have not been 'vaccinated'. Madej speculated that perhaps it was 5G and increased levels of other radiation that was causing the symptoms directly although interestingly she said that non-'vaccinated' patients had shown improvement when they were away from the 'vaccinated' person they had interacted with. It must be remembered that you can control frequency and energy with your mind and you can consciously create energetic barriers or bubbles with the mind to stop damaging frequencies from penetrating your field. American paediatrician Dr Larry Palevsky said the 'vaccine' was not a 'vaccine' and was never designed to protect from a 'viral' infection. He called it 'a massive, brilliant propaganda of genocide' because they didn't have to inject everyone to get the result they wanted. He said the content of the jabs was able to infuse any material into the brain, heart, lungs, kidneys, liver, sperm and female productive system. 'This is genocide; this is a weapon of mass destruction.' At the same time American colleges were banning students from attending if they didn't have this life-changing and potentially life-ending 'vaccine'. Class action lawsuits must follow when the consequences of this college fascism come to light. As the book was going to press came reports about fertility effects on sperm in 'vaccinated' men which would absolutely fit with what I have been saying and hospitals continued to fill with 'vaccine' reactions. Another question is what about transmission via blood transfusions? The NHS has extended blood donation restrictions from seven days after a 'Covid vaccination' to 28 days after even a sore arm reaction.

I said in the spring of 2020 that the then touted 'Covid vaccine' would be ongoing each year like the flu jab. A year later Pfizer CEO, the appalling Albert Bourla, said people would 'likely' need a 'booster dose' of the 'vaccine' within 12 months of getting 'fully

vaccinated' and then a yearly shot. 'Variants will play a key role', he said confirming the point. Johnson & Johnson CEO Alex Gorsky also took time out from his 'vaccine' disaster to say that people may need to be vaccinated against 'Covid-19' each year. UK Health Secretary, the psychopath Matt Hancock, said additional 'boosters' would be available in the autumn of 2021. This is the trap of the 'vaccine passport'. The public will have to accept every last 'vaccine' they introduce, including for the fake 'variants', or it would cease to be valid. The only other way in some cases would be continuous testing with a test not testing for the 'virus' and what is on the swabs constantly pushed up your noise towards the brain every time?

'Vaccines' changing behaviour

I mentioned in the body of the book how I believed we would see gathering behaviour changes in the 'vaccinated' and I am already hearing such comments from the non-'vaccinated' describing behaviour changes in friends, loved ones and work colleagues. This will only increase as the self-replicating synthetic material and nanoparticles expand in body and brain. An article in the *Guardian* in 2016 detailed research at the University of Virginia in Charlottesville which developed a new method for controlling brain circuits associated with complex animal behaviour. The method, dubbed 'magnetogenetics', involves genetically-engineering a protein called ferritin, which stores and releases iron, to create a magnetised substance – 'Magneto' – that can activate specific groups of nerve cells from a distance. This is claimed to be an advance on other methods of brain activity manipulation known as optogenetics and chemogenetics (the Cult has been developing methods of brain control for a long time). The ferritin technique is said to be non-invasive and able to activate neurons 'rapidly and reversibly'. In other words, human thought and perception. The article said that earlier studies revealed how nerve cell proteins 'activated by heat and mechanical pressure can be genetically engineered so that they become sensitive to radio waves and magnetic fields, by attaching them to an iron-storing protein called ferritin, or to inorganic

paramagnetic particles'. Sensitive to radio waves and magnetic fields? You mean like 5G, 6G and 7G? This is the human-AI Smart Grid hive mind we are talking about. The *Guardian* article said:

> … the researchers injected Magneto into the striatum of freely behaving mice, a deep brain structure containing dopamine-producing neurons that are involved in reward and motivation, and then placed the animals into an apparatus split into magnetised and non-magnetised sections.
>
> Mice expressing Magneto spent far more time in the magnetised areas than mice that did not, because activation of the protein caused the striatal neurons expressing it to release dopamine, so that the mice found being in those areas rewarding. This shows that Magneto can remotely control the firing of neurons deep within the brain, and also control complex behaviours.

Make no mistake this basic methodology will be part of the 'Covid vaccine' cocktail and using magnetics to change brain function through electromagnetic field frequency activation. The Pentagon is developing a 'Covid vaccine' using ferritin. Magnetics would explain changes in behaviour and why videos are appearing across the Internet as I write showing how magnets stick to the skin at the point of the 'vaccine' shot. Once people take these 'vaccines' anything becomes possible in terms of brain function and illness which will be blamed on 'Covid-19' and 'variants'. Magnetic field manipulation would further explain why the non-'vaccinated' are reporting the same symptoms as the 'vaccinated' they interact with and why those symptoms are reported to decrease when not in their company. Interestingly 'Magneto', a 'mutant', is a character in the Marvel Comic *X-Men* stories with the ability to manipulate magnetic fields and he believes that mutants should fight back against their human oppressors by any means necessary. The character was born Erik Lehnsherr to a Jewish family in Germany.

Cult-controlled courts

The European Court of Human Rights opened the door for mandatory 'Covid-19 vaccines' across the continent when it ruled in a Czech Republic dispute over childhood immunisation that legally

enforced vaccination could be 'necessary in a democratic society'. The 17 judges decided that compulsory vaccinations did not breach human rights law. On the face of it the judgement was so inverted you gasp for air. If not having a vaccine infused into your body is not a human right then what is? Ah, but they said human rights law which has been specifically written to delete all human rights at the behest of the state (the Cult). Article 8 of the European Convention on Human Rights relates to the right to a private life. The crucial word here is *'except'*:

> There shall be no interference by a public authority with the exercise of this right EXCEPT such as is in accordance with the law and is necessary in a democratic society in the interests of national security, public safety or the economic wellbeing of the country, for the prevention of disorder or crime, for the protection of health or morals, or for the protection of the rights and freedoms of others [My emphasis].

No interference *except* in accordance with the law means there *are* no 'human rights' *except* what EU governments decide you can have at their behest. 'As is necessary in a democratic society' explains that reference in the judgement and 'in the interests of national security, public safety or the economic well-being of the country, for the prevention of disorder or crime, for the protection of health or morals, or for the protection of the rights and freedoms of others' gives the EU a coach and horses to ride through 'human rights' and scatter them in all directions. The judiciary is not a check and balance on government extremism; it is a vehicle to enforce it. This judgement was almost laughably predictable when the last thing the Cult wanted was a decision that went against mandatory vaccination. Judges rule over and over again to benefit the system of which they are a part. Vaccination disputes that come before them are invariably delivered in favour of doctors and authorities representing the view of the state which owns the judiciary. Oh, yes, and we have even had calls to stop putting 'Covid-19' on death certificates within 28 days of a 'positive test' because it is claimed the practice makes the 'vaccine' appear not to work. They are laughing at you.

The scale of madness, inhumanity and things to come was highlighted when those not 'vaccinated' for 'Covid' were refused evacuation from the Caribbean island of St Vincent during massive volcanic eruptions. Cruise ships taking residents to the safety of another island allowed only the 'vaccinated' to board and the rest were left to their fate. Even in life and death situations like this we see 'Covid' stripping people of their most basic human instincts and the insanity is even more extreme when you think that fake 'vaccine'-makers are not even claiming their body-manipulating concoctions stop 'infection' and 'transmission' of a 'virus' that doesn't exist. St Vincent Prime Minister Ralph Gonsalves said: 'The chief medical officer will be identifying the persons already vaccinated so that we can get them on the ship.' Note again the power of the chief medical officer who, like Whitty in the UK, will be answering to the World Health Organization. This is the Cult network structure that has overridden politicians who 'follow the science' which means doing what WHO-controlled 'medical officers' and 'science advisers' tell them. Gonsalves even said that residents who were 'vaccinated' after the order so they could board the ships would still be refused entry due to possible side effects such as 'wooziness in the head'. The good news is that if they were woozy enough in the head they could qualify to be prime minister of St Vincent.

Microchipping freedom

The European judgement will be used at some point to justify moves to enforce the 'Covid' DNA-manipulating procedure. Sandra Ro, CEO of the Global Blockchain Business Council, told a World Economic Forum event that she hoped 'vaccine passports' would help to 'drive forced consent and standardisation' of global digital identity schemes: 'I'm hoping with the desire and global demand for some sort of vaccine passport – so that people can get travelling and working again – [it] will drive forced consent, standardisation, and frankly, cooperation across the world.' The lady is either not very bright, or thoroughly mendacious, to use the term 'forced consent'.

You do not 'consent' if you are forced – you *submit*. She was describing what the plan has been all along and that's to enforce a digital identity on every human without which they could not function. 'Vaccine passports' are opening the door and are far from the end goal. A digital identity would allow you to be tracked in everything you do in cyberspace and this is the same technique used by Cult-owned China to enforce its social credit system of total control. The ultimate 'passport' is planned to be a microchip as my books have warned for nearly 30 years. Those nice people at the Pentagon working for the Cult-controlled Defense Advanced Research Projects Agency (DARPA) claimed in April, 2021, they have developed a microchip inserted under the skin to detect 'asymptomatic Covid-19 infection' before it becomes an outbreak and a 'revolutionary filter' that can remove the 'virus' from the blood when attached to a dialysis machine. The only problems with this are that the 'virus' does not exist and people transmitting the 'virus' with no symptoms is brain-numbing bullshit. This is, of course, not a ruse to get people to be microchipped for very different reasons. DARPA also said it was producing a one-stop 'vaccine' for the 'virus' and all 'variants'. One of the most sinister organisations on Planet Earth is doing this? Better have it then. These people are insane because Wetiko that possesses them is insane.

Researchers from the Salk Institute in California announced they have created an embryo that is part human and part monkey. My books going back to the 1990s have exposed experiments in top secret underground facilities in the United States where humans are being crossed with animal and non-human 'extraterrestrial' species. They are now easing that long-developed capability into the public arena and there is much more to come given we are dealing with psychiatric basket cases. Talking of which – Elon Musk's scientists at Neuralink trained a monkey to play Pong and other puzzles on a computer screen using a joystick and when the monkey made the correct move a metal tube squirted banana smoothie into his mouth which is the basic technique for training humans into unquestioning compliance. Two Neuralink chips were in the monkey's skull and

more than 2,000 wires 'fanned out' into its brain. Eventually the monkey played a video game purely with its brain waves. Psychopathic narcissist Musk said the 'breakthrough' was a step towards putting Neuralink chips into human skulls and merging minds with artificial intelligence. *Exactly*. This man is so dark and Cult to his DNA.

World Economic Fascism (WEF)

The World Economic Forum is telling you the plan by the statements made at its many and various events. Cult-owned fascist YouTube CEO Susan Wojcicki spoke at the 2021 WEF Global Technology Governance Summit (see the name) in which 40 governments and 150 companies met to ensure 'the responsible design and deployment of emerging technologies'. Orwellian translation: 'Ensuring the design and deployment of long-planned technologies will advance the Cult agenda for control and censorship.' Freedom-destroyer and Nuremberg-bound Wojcicki expressed support for tech platforms like hers to censor content that is 'technically legal but could be harmful'. Who decides what is 'harmful'? She does and they do. 'Harmful' will be whatever the Cult doesn't want people to see and we have legislation proposed by the UK government that would censor content on the basis of 'harm' no matter if the information is fair, legal and provably true. Make that *especially* if it is fair, legal and provably true. Wojcicki called for a global coalition to be formed to enforce content moderation standards through automated censorship. This is a woman and mega-censor so self-deluded that she shamelessly accepted a 'free expression' award – *Wojcicki* – in an event sponsored by her own *YouTube*. They have no shame and no self-awareness.

You know that 'Covid' is a scam and Wojcicki a Cult operative when YouTube is censoring medical and scientific opinion purely on the grounds of whether it supports or opposes the Cult 'Covid' narrative. Florida governor Ron DeSantis compiled an expert panel with four professors of medicine from Harvard, Oxford, and Stanford Universities who spoke against forcing children and

vaccinated people to wear masks. They also said there was no proof that lockdowns reduced spread or death rates of 'Covid-19'. Cult-gofer Wojcicki and her YouTube deleted the panel video 'because it included content that contradicts the consensus of local and global health authorities regarding the efficacy of masks to prevent the spread of Covid-19'. This 'consensus' refers to what the Cult tells the World Health Organization to say and the WHO tells 'local health authorities' to do. Wojcicki knows this, of course. The panellists pointed out that censorship of scientific debate was responsible for deaths from many causes, but Wojcicki couldn't care less. She would not dare go against what she is told and as a disgrace to humanity she wouldn't want to anyway. The UK government is seeking to pass a fascist 'Online Safety Bill' to specifically target with massive fines and other means non-censored video and social media platforms to make them censor 'lawful but harmful' content like the Cult-owned Facebook, Twitter, Google and YouTube. What is 'lawful but harmful' would be decided by the fascist Blair-created Ofcom.

Another WEF obsession is a cyber-attack on the financial system and this is clearly what the Cult has planned to take down the bank accounts of everyone – except theirs. Those that think they have enough money for the Cult agenda not to matter to them have got a big lesson coming if they continue to ignore what is staring them in the face. The World Economic Forum, funded by Gates and fronted by Klaus Schwab, announced it would be running a 'simulation' with the Russian government and global banks of just such an attack called Cyber Polygon 2021. What they simulate – as with the 'Covid' Event 201 – they plan to instigate. The WEF is involved in a project with the Cult-owned Carnegie Endowment for International Peace called the WEF-Carnegie Cyber Policy Initiative which seeks to merge Wall Street banks, 'regulators' (I love it) and intelligence agencies to 'prevent' (arrange and allow) a cyber-attack that would bring down the global financial system as long planned by those that control the WEF and the Carnegie operation. The Carnegie Endowment for International Peace sent an instruction to First World

War US President Woodrow Wilson not to let the war end before society had been irreversibly transformed.

The Wuhan lab diversion

As I close, the Cult-controlled authorities and lapdog media are systematically pushing 'the virus was released from the Wuhan lab' narrative. There are two versions – it happened by accident and it happened on purpose. Both are nonsense. The perceived existence of the never-shown-to-exist 'virus' is vital to sell the impression that there is actually an infective agent to deal with and to allow the endless potential for terrifying the population with 'variants' of a 'virus' that does not exist. The authorities at the time of writing are going with the 'by accident' while the alternative media is promoting the 'on purpose'. Cable news host Tucker Carlson who has questioned aspects of lockdown and 'vaccine' compulsion has bought the Wuhan lab story. 'Everyone now agrees' he said. Well, I don't and many others don't and the question is *why* does the system and its media suddenly 'agree'? When the media moves as one unit with a narrative it is always a lie – witness the hour by hour mendacity of the 'Covid' era. Why would this Cult-owned combination which has unleashed lies like machine gun fire suddenly 'agree' to tell the truth??

Much of the alternative media is buying the lie because it fits the conspiracy narrative, but it's the *wrong* conspiracy. The real conspiracy is that *there is no virus* and that is what the Cult is desperate to hide. The idea that the 'virus' was released by accident is ludicrous when the whole 'Covid' hoax was clearly long-planned and waiting to be played out as it was so fast in accordance with the Rockefeller document and Event 201. So they prepared everything in detail over decades and then sat around strumming their fingers waiting for an 'accidental' release from a bio-lab? *What??* It's crazy. Then there's the 'on purpose' claim. You want to circulate a 'deadly virus' and hide the fact that you've done so and you release it down the street from the highest-level bio-lab in China? I repeat – *What??*

You would release it far from that lab to stop any association being made. But, no, we'll do it in a place where the connection was certain to be made. Why would you need to scam 'cases' and 'deaths' and pay hospitals to diagnose 'Covid-19' if you had a real 'virus'? What are sections of the alternative media doing believing this crap? Where were all the mass deaths in Wuhan from a 'deadly pathogen' when the recovery to normal life after the initial propaganda was dramatic in speed? Why isn't the 'deadly pathogen' now circulating all over China with bodies in the street? Once again we have the technique of tell them what they want to hear and they will likely believe it. The alternative media has its 'conspiracy' and with Carlson it fits with his 'China is the danger' narrative over years. China *is* a danger as a global Cult operations centre, but not for this reason. The Wuhan lab story also has the potential to instigate conflict with China when at some stage the plan is to trigger a Problem-Reaction-Solution confrontation with the West. Question everything – *everything* – and especially when the media agrees on a common party line.

Third wave ... fourth wave ... fifth wave ...

As the book went into production the world was being set up for more lockdowns and a 'third wave' supported by invented 'variants' that were increasing all the time and will continue to do so in public statements and computer programs, but not in reality. India became the new Italy in the 'Covid' propaganda campaign and we were told to be frightened of the new 'Indian strain'. Somehow I couldn't find it within myself to do so. A document produced for the UK government entitled 'Summary of further modelling of easing of restrictions – Roadmap Step 2' declared that a third wave was inevitable (of course when it's in the script) and it would be the fault of children and those who refuse the health-destroying fake 'Covid vaccine'. One of the computer models involved came from the Cult-owned *Imperial College* and the other from Warwick University which I wouldn't trust to tell me the date in a calendar factory. The document states that both models presumed extremely high uptake

of the 'Covid vaccines' and didn't allow for 'variants'. The document states: 'The resurgence is a result of some people (mostly children) being ineligible for vaccination; others choosing not to receive the vaccine; and others being vaccinated but not perfectly protected.' The mendacity takes the breath away. Okay, blame those with a brain who won't take the DNA-modifying shots and put more pressure on children to have it as 'trials' were underway involving children as young as six months with parents who give insanity a bad name. Massive pressure is being put on the young to have the fake 'vaccine' and child age consent limits have been systematically lowered around the world to stop parents intervening. Most extraordinary about the document was its claim that the 'third wave' would be driven by 'the resurgence in both hospitalisations and deaths … dominated by *those that have received two doses of the vaccine*, comprising around 60-70% of the wave respectively'. The predicted peak of the 'third wave' suggested 300 deaths per day with 250 of them *fully 'vaccinated' people*. How many more lies do acquiescers need to be told before they see the obvious? Those who took the jab to 'protect themselves' are projected to be those who mostly get sick and die? So what's in the 'vaccine'? The document went on:

> It is possible that a summer of low prevalence could be followed by substantial increases in incidence over the following autumn and winter. Low prevalence in late summer should not be taken as an indication that SARS-CoV-2 has retreated or that the population has high enough levels of immunity to prevent another wave.

They are telling you the script and while many British people believed 'Covid' restrictions would end in the summer of 2021 the government was preparing for them to be ongoing. Authorities were awarding contracts for 'Covid marshals' to police the restrictions with contracts starting in July, 2021, and going through to January 31st, 2022, and the government was advertising for 'Media Buying Services' to secure media propaganda slots worth a potential £320 million for 'Covid-19 campaigns' with a contract not ending until March, 2022. The recipient – via a list of other front companies – was reported to be American media marketing giant Omnicom Group

Inc. While money is no object for 'Covid' the UK waiting list for all other treatment – including life-threatening conditions – passed 4.5 million. Meantime the Cult is seeking to control all official 'inquiries' to block revelations about what has really been happening and why. It must not be allowed to – we need Nuremberg jury trials in every country. The cover-up doesn't get more obvious than appointing ultra-Zionist professor Philip Zelikow to oversee two dozen US virologists, public health officials, clinicians, former government officials and four American 'charitable foundations' to 'learn the lessons' of the 'Covid' debacle. The personnel will be those that created and perpetuated the 'Covid' lies while Zelikow is the former executive director of the 9/11 Commission who ensured that the truth about those attacks never came out and produced a report that must be among the most mendacious and manipulative documents ever written – see *The Trigger* for the detailed exposure of the almost unimaginable 9/11 story in which Sabbatians can be found at every level.

Passive no more

People are increasingly challenging the authorities with amazing numbers of people taking to the streets in London well beyond the ability of the Face-Nappies to stop them. Instead the Nappies choose situations away from the mass crowds to target, intimidate, and seek to promote the impression of 'violent protestors'. One such incident happened in London's Hyde Park. Hundreds of thousands walking through the streets in protest against 'Covid' fascism were ignored by the Cult-owned BBC and most of the rest of the mainstream media, but they delighted in reporting how police were injured in 'clashes with protestors'. The truth was that a group of people gathered in Hyde Park at the end of one march when most had gone home and they were peacefully having a good time with music and chat. Face-Nappies who couldn't deal with the full-march crowd then waded in with their batons and got more than they bargained for. Instead of just standing for this criminal brutality the crowd used their numerical superiority to push the Face-Nappies out of the

park. Eventually the Nappies turned and ran. Unfortunately two or three idiots in the crowd threw drink cans striking two officers which gave the media and the government the image they wanted to discredit the 99.9999 percent who were peaceful. The idiots walked straight into the trap and we must always be aware of potential agent provocateurs used by the authorities to discredit their targets.

This response from the crowd – the can people apart – must be a turning point when the public no longer stand by while the innocent are arrested and brutally attacked by the Face-Nappies. That doesn't mean to be violent, that's the last thing we need. We'll leave the violence to the Face-Nappies and government. But it does mean that when the Face-Nappies use violence against peaceful people the numerical superiority is employed to stop them and make citizen's arrests or Common Law arrests for a breach of the peace. The time for being passive in the face of fascism is over.

We are the many, they are the few, and we need to make that count before there is no freedom left and our children and grandchildren face an ongoing fascist nightmare.

COME ON PEOPLE – IT'S TIME.

One final thought …

The power of love
A force from above
Cleaning my soul
Flame on burn desire
Love with tongues of fire
Purge the soul
Make love your goal

I'll protect you from the hooded claw
Keep the vampires from your door
When the chips are down I'll be around
With my undying, death-defying
Love for you

Envy will hurt itself
Let yourself be beautiful
Sparkling love, flowers
And pearls and pretty girls
Love is like an energy
Rushin' rushin' inside of me

This time we go sublime
Lovers entwine, divine, divine,
Love is danger, love is pleasure
Love is pure – the only treasure

I'm so in love with you
Purge the soul
Make love your goal

The power of love
A force from above
Cleaning my soul
The power of love
A force from above
A sky-scraping dove

Flame on burn desire
Love with tongues of fire
Purge the soul
Make love your goal

Frankie Goes To Hollywood

APPENDIX

Cowan-Kaufman-Morell Statement on Virus Isolation (SOVI)

Isolation: The action of isolating; the fact or condition of being isolated or standing alone; separation from other things or persons; solitariness
Oxford English Dictionary

The controversy over whether the SARS-CoV-2 virus has ever been isolated or purified continues. However, using the above definition, common sense, the laws of logic and the dictates of science, any unbiased person must come to the conclusion that the SARS-CoV-2 virus has never been isolated or purified. As a result, no confirmation of the virus' existence can be found. The logical, common sense, and scientific consequences of this fact are:

- the structure and composition of something not shown to exist can't be known, including the presence, structure, and function of any hypothetical spike or other proteins;
- the genetic sequence of something that has never been found can't be known;
- "variants" of something that hasn't been shown to exist can't be known;
- it's impossible to demonstrate that SARS-CoV-2 causes a disease called Covid-19.

In as concise terms as possible, here's the proper way to isolate, characterize and demonstrate a new virus. First, one takes samples (blood, sputum, secretions) from many people (e.g. 500) with symptoms which are unique and specific enough to characterize an illness. Without mixing these samples with ANY tissue or products that also contain genetic material, the virologist macerates, filters and ultracentrifuges i.e. *purifies* the specimen. This common virology technique, done for decades to isolate bacteriophages[1] and so-called giant viruses in every virology lab, then allows the virologist to demonstrate with electron microscopy thousands of identically sized and shaped particles. These particles are the isolated and purified virus.

These identical particles are then checked for uniformity by physical and/or microscopic techniques. Once the purity is determined, the particles may be further characterized. This would include examining the structure, morphology, and chemical composition of the particles. Next, their genetic makeup is characterized by extracting the genetic material directly from the purified particles and using genetic-sequencing techniques, such as Sanger sequencing, that have also been around for decades. Then one does an analysis to confirm that these uniform particles are exogenous (outside) in origin as a virus is conceptualized to be, and not the normal breakdown products of dead and dying tissues.[2] (As of May 2020, we know that virologists have no way to determine whether the particles they're seeing are viruses or just normal breakdown products of dead and dying tissues.)[3]

---

1 Isolation, characterization and analysis of bacteriophages from the haloalkaline lake Elmenteita, KenyaJuliah Khayeli Akhwale et al, PLOS One, Published: April 25, 2019. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0215734 – accessed 2/15/21

2 "Extracellular Vesicles Derived From Apoptotic Cells: An Essential Link Between Death and Regeneration," Maojiao Li1 et al, Frontiers in Cell and Developmental Biology, 2020 October 2. https://www.frontiersin.org/articles/10.3389/fcell.2020.573511/full – accessed 2/15/21

3 "The Role of Extraellular Vesicles as Allies of HIV, HCV and SARS Viruses," Flavia Giannessi, et al, Viruses, 2020 May

If we have come this far then we have fully isolated, characterized, and genetically sequenced an exogenous virus particle. However, we still have to show it is causally related to a disease. This is carried out by exposing a group of healthy subjects (animals are usually used) to this isolated, purified virus in the manner in which the disease is thought to be transmitted. If the animals get sick with the same disease, as confirmed by clinical and autopsy findings, one has now shown that the virus actually causes a disease. This demonstrates infectivity and transmission of an infectious agent.

None of these steps has even been attempted with the SARS-CoV-2 virus, nor have all these steps been successfully performed for any so-called pathogenic virus. Our research indicates that a single study showing these steps does not exist in the medical literature.

Instead, since 1954, virologists have taken unpurified samples from a relatively few people, often less than ten, with a similar disease. They then minimally process this sample and inoculate this unpurified sample onto tissue culture containing usually four to six other types of material – all of which contain identical genetic material as to what is called a "virus." The tissue culture is starved and poisoned and naturally disintegrates into many types of particles, some of which contain genetic material. Against all common sense, logic, use of the English language and scientific integrity, this process is called "virus isolation." This brew containing fragments of genetic material from many sources is then subjected to genetic analysis, which then creates in a computer-simulation process the alleged sequence of the alleged virus, a so called in silico genome. At no time is an actual virus confirmed by electron microscopy. At no time is a genome extracted and sequenced from an actual virus. This is scientific fraud.

The observation that the unpurified specimen — inoculated onto tissue culture along with toxic antibiotics, bovine fetal tissue, amniotic fluid and other tissues — destroys the kidney tissue onto which it is inoculated is given as evidence of the virus' existence and pathogenicity. This is scientific fraud.

From now on, when anyone gives you a paper that suggests the SARS-CoV-2 virus has been isolated, please check the methods sections. If the researchers used Vero cells or any other culture method, you know that their process was not isolation. You will hear the following excuses for why actual isolation isn't done:

1. There were not enough virus particles found in samples from patients to analyze.

2. Viruses are intracellular parasites; they can't be found outside the cell in this manner.

If No. 1 is correct, and we can't find the virus in the sputum of sick people, then on what evidence do we think the virus is dangerous or even lethal? If No. 2 is correct, then how is the virus spread from person to person? We are told it emerges from the cell to infect others. Then why isn't it possible to find it?

Finally, questioning these virology techniques and conclusions is not some distraction or divisive issue. Shining the light on this truth is essential to stop this terrible fraud that humanity is confronting. For, as we now know, if the virus has never been isolated, sequenced or shown to cause illness, if the virus is imaginary, then why are we wearing masks, social distancing and putting the whole world into prison?

Finally, if pathogenic viruses don't exist, then what is going into those injectable devices erroneously called "vaccines," and what is their purpose? This scientific question is the most urgent and relevant one of our time.

We are correct. The SARS-CoV2 virus does not exist.

Sally Fallon Morell, MA
Dr. Thomas Cowan, MD
Dr. Andrew Kaufman, MD

Bibliography

Alinsky, Saul: *Rules for Radicals* (Vintage, 1989)

Antelman, Rabbi Marvin: *To Eliminate the Opiate* (Zahavia, 1974)

Bastardi, Joe: *The Climate Chronicles* (Relentless Thunder Press, 2018)

Cowan, Tom: *Human Heart, Cosmic Heart* (Chelsea Green Publishing, 2016)

Cowan, Tom, and Fallon Morell, Sally: *The Contagion Myth* (Skyhorse Publishing, 2020)

Forbes, Jack D: *Columbus And Other Cannibals – The Wetiko Disease of Exploitation, Imperialism, and Terrorism* (Seven Stories Press, 2008 – originally published in 1979)

Gates, Bill: *How to Avoid a Climate Disaster: The Solutions We Have and the Breakthroughs We Need* (Allen Lane, 2021)

Huxley, Aldous: *Brave New World* (Chatto & Windus, 1932)

Köhnlein, Dr Claus, and Engelbrecht, Torsten: *Virus Mania* (emu-Vertag, Lahnstein, 2020)

Lanza, Robert, and Berman, Bob: *Biocentrism* (BenBella Books, 2010)

Lash, John Lamb: *Not In His Image* (Chelsea Green Publishing, 2006)

Lester, Dawn, and Parker, David: *What Really Makes You Ill – Why everything you thought you knew about disease is wrong* (Independently Published, 2019)

Levy, Paul: *Dispelling Wetiko, Breaking the Spell of Evil* (North Atlantic Books, 2013)

Marx, Karl: *A World Without Jews* (Philosophical Library, first edition, 1959)

Mullis, Kary: *Dancing Naked in the Mine Field* (Bloomsbury, 1999)

O'Brien, Cathy: *Trance-Formation of America* (Reality Marketing, 1995)

Scholem, Gershon: *The Messianic Idea in Judaism* (Schocken Books, 1994)

Schwab, Klaus, and Davis, Nicholas: *Shaping the Future of the Fourth Industrial Revolution: A guide to building a better world* (Penguin Books, 2018)

Schwab, Klaus: *The Great Reset* (Agentur Schweiz, 2020)

Sunstein, Cass and Thaler, Richard: *Nudge: Improving Decisions About Health, Wealth, and Happiness* (Penguin, 2009)

Swan, Shanna: *Count Down: How Our Modern World Is Threatening Sperm Counts, Altering Male and Female Reproductive Development and Imperiling the Future of the Human Race* (Scribner, 2021)

Tegmark, Max: *Our Mathematical Universe: My Quest for the Ultimate Nature of Reality* (Penguin, 2015)

Velikovsky, Immanuel: *Worlds in Collision* (Paradigma, 2009)

Wilton, Robert: *The Last Days of the Romanovs* (Blurb, 2018, first published 1920)

Index

A

abusive relationships
blaming themselves, abused as ref1
children ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10
conspiracy theories ref1
domestic abuse ref1, ref2
economic abuse and dependency ref1
isolation ref1
physical abuse ref1
psychological abuse ref1
signs of abuse ref1
addiction
alcoholism ref1
frequencies ref1
substance abuse ref1, ref2
technology ref1, ref2, ref3
Adelson, Sheldon ref1, ref2, ref3
Agenda 21/Agenda 2030 (UN) ref1, ref2, ref3, ref4
AIDs/HIV ref1
causal link between HIV and AIDs ref1, ref2
retroviruses ref1
testing ref1, ref2
trial-run for Covid-19, as ref1, ref2
aliens/extraterrestrials ref1, ref2
aluminium ref1
Amazon ref1, ref2, ref3

amplification cycles ref1, ref2
anaphylactic shock ref1, ref2, ref3, ref4
animals ref1, ref2, ref3
antibodies ref1, ref2, ref3, ref4, ref5
Antifa ref1, ref2, ref3, ref4
antigens ref1, ref2
anti-Semitism ref1, ref2, ref3
Archons ref1, ref2
  consciousness ref1, ref2, ref3
  energy ref1, ref2, ref3
  ennoia ref1
  genetic manipulation ref1, ref2
  inversion ref1, ref2, ref3
  lockdowns ref1
  money ref1
  radiation ref1
  religion ref1, ref2
  technology ref1, ref2, ref3
  Wetiko factor ref1, ref2, ref3, ref4
artificial intelligence (AI) ref1
army made up of robots ref1, ref2
  Human 2.0 ref1, ref2
  Internet ref1
  MHRA ref1
  Morgellons fibres ref1, ref2
  Smart Grid ref1
  Wetiko factor ref1
asymptomatic, Covid-19 as ref1, ref2, ref3
aviation industry ref1

B

banking, finance and money ref1, ref2, ref3
2008 crisis ref1, ref2
boom and bust ref1
cashless digital money systems ref1
central banks ref1
credit ref1
digital currency ref1
fractional reserve lending ref1
Great Reset ref1
guaranteed income ref1, ref2, ref3
Human 2.0 ref1
incomes, destruction of ref1, ref2
interest ref1
one per cent ref1, ref2
scams ref1
BBC ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
Becker-Phelps, Leslie ref1
Behavioural Insights Team (BIT) (Nudge Unit) ref1, ref2, ref3
behavioural scientists *and* **psychologists, advice from** ref1, ref2
Bezos, Jeff ref1, ref2, ref3, ref4
Biden, Hunter ref1
Biden, Joe ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10, ref11, ref12, ref13, ref14, ref15, ref16, ref17
Big Pharma
cholesterol ref1
health professionals ref1, ref2
immunity from prosecution in US ref1
vaccines ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
Wetiko factor ref1, ref2
WHO ref1, ref2, ref3
Bill and Melinda Gates Foundation ref1, ref2, ref3, ref4, ref5, ref6, ref7

billionaires ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9 ref10, ref11
bird flu (H5N1) ref1
Black Lives Matter (BLM) ref1, ref2, ref3, ref4, ref5
Blair, Tony ref1, ref2, ref3, ref4, ref5, ref6, ref7
Brin, Sergei ref1, ref2, ref3, ref4, ref5, ref6, ref7
British Empire ref1
Bush, George HW ref1, ref2
Bush, George W ref1, ref2, ref3, ref4
Byrd, Robert ref1

C

Canada
- Global Cult ref1
- hate speech ref1
- internment ref1
- masks ref1
- old people ref1
- SARS-COV-2 ref1
- satellites ref1
- vaccines ref1
- wearable technology ref1

Capitol Hill riot ref1, ref2
- agents provocateur ref1
- Antifa ref1
- Black Lives Matter (BLM) ref1, ref2
- QAnon ref1
- security precautions, lack of ref1, ref2, ref3

carbon dioxide ref1, ref2
care homes, deaths in ref1, ref2
cashless digital money systems ref1
censorship ref1, ref2, ref3, ref4, ref5

fact-checkers ref1
masks ref1
media ref1, ref2
private messages ref1
social media ref1, ref2, ref3, ref4, ref5, ref6
transgender persons ref1
vaccines ref1, ref2, ref3
Wokeness ref1
Centers for Disease Control (CDC) (United States) ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10, ref11, ref12, ref13
centralisation ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
chakras ref1
change agents ref1, ref2, ref3
chemtrails ref1, ref2, ref3
chief medical officers and scientific advisers ref1, ref2, ref3, ref4, ref5, ref6
children *see also* **young people**
abuse ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10
care, taken into ref1, ref2, ref3
education ref1, ref2, ref3, ref4
energy ref1
family courts ref1
hand sanitisers ref1
human sacrifice ref1
lockdowns ref1, ref2, ref3
masks ref1, ref2, ref3, ref4, ref5
mental health ref1
old people ref1
parents, replacement of ref1, ref2
Psyop (psychological operation), Covid as a ref1, ref2
reframing ref1
smartphone addiction ref1

social distancing and isolation ref1
social media ref1
transgender persons ref1, ref2
United States ref1
vaccines ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10
Wetiko factor ref1
China ref1, ref2, ref3, ref4
anal swab tests ref1
Chinese Revolution ref1, ref2, ref3
digital currency ref1
Global Cult ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9
guaranteed income ref1
Imperial College ref1
Israel ref1
lockdown ref1, ref2
masculinity crisis ref1
masks ref1
media ref1
origins of virus in China ref1, ref2, ref3, ref4, ref5
pollution causing respiratory diseases ref1
Sabbatians ref1, ref2
Smart Grid ref1, ref2
social credit system ref1
testing ref1, ref2
United States ref1, ref2
vaccines ref1, ref2
Wetiko factor ref1
wet market conspiracy ref1
Wuhan ref1, ref2, ref3, ref4, ref5, ref6, ref7
cholesterol ref1, ref2
Christianity ref1, ref2, ref3, ref4, ref5
criticism ref1
cross, inversion of the ref1

Nag Hammadi texts ref1, ref2, ref3
Roman Catholic Church ref1, ref2
Sabbatians ref1, ref2
Satan ref1, ref2, ref3, ref4
Wokeness ref1
class ref1, ref2
climate change hoax ref1, ref2, ref3, ref4, ref5
Agenda 21/Agenda 2030 ref1, ref2, ref3
carbon dioxide ref1, ref2
Club of Rome ref1, ref2, ref3, ref4, ref5
fear ref1
funding ref1
Global Cult ref1
green new deals ref1
green parties ref1
inversion ref1
perception, control of ref1
PICC ref1
reframing ref1
temperature, increases in ref1
United Nations ref1, ref2
Wikipedia ref1
Wokeness ref1, ref2
Clinton, Bill ref1, ref2, ref3, ref4, ref5, ref6
Clinton, Hillary ref1, ref2, ref3
the cloud ref1, ref2, ref3, ref4, ref5, ref6, ref7
Club of Rome and climate change hoax ref1, ref2, ref3, ref4, ref5
cognitive therapy ref1
Cohn, Roy ref1
Common Law ref1
Admiralty Law ref1
arrests ref1, ref2

contractual law, Statute Law as ref1
corporate entities, people as ref1
legalese ref1
sea, law of the ref1
Statute Law ref1
Common Purpose leadership programme ref1, ref2
communism ref1, ref2
co-morbidities ref1
computer-generated virus,
Covid-19 as ref1, ref2, ref3
computer models ref1, ref2, ref3, ref4, ref5
connections ref1, ref2, ref3, ref4
consciousness ref1, ref2, ref3, ref4
Archons ref1, ref2, ref3
expanded ref1, ref2, ref3, ref4, ref5, ref6, ref7
experience ref1
heart ref1
infinity ref1, ref2
religion ref1, ref2
self-identity ref1
simulation thesis ref1
vaccines ref1
Wetiko factor ref1, ref2
conspiracy theorists ref1, ref2, ref3, ref4, ref5
contradictory rules ref1
contrails ref1
Corman-Drosten test ref1, ref2, ref3, ref4
countermimicry ref1, ref2, ref3
Covid-19 vaccines *see* vaccines
Covidiots ref1, ref2
Cowan, Tom ref1, ref2, ref3, ref4
crimes against humanity ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8

cyber-operations ref1
cyberwarfare ref1

D

DARPA (Defense Advanced Research Projects Agency) ref1
deaths
  care homes ref1
  certificates ref1, ref2, ref3, ref4
  mortality rate ref1
  post-mortems/autopsies ref1
  recording ref1, ref2, ref3, ref4, ref5, ref6, ref7
  vaccines ref1, ref2, ref3, ref4, ref5
deceit
  pyramid of deceit ref1, ref2
  sequence of deceit ref1
decoding ref1, ref2, ref3
dehumanisation ref1, ref2, ref3
Delphi technique ref1
democracy ref1
dependency ref1, ref2, ref3, ref4, ref5
Descartes, René ref1
DNA
  numbers ref1
  vaccines ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10
DNR (do not resuscitate)
  orders ref1
domestic abuse ref1, ref2
downgrading of Covid-19 ref1
Drosten, Christian ref1, ref2, ref3, ref4, ref5, ref6, ref7
Duesberg, Peter ref1, ref2

E

economic abuse ref1
Edmunds, John ref1, ref2
education ref1, ref2, ref3, ref4
electromagnetic spectrum ref1, ref2
Enders, John ref1
energy
  Archons ref1, ref2, ref3
  children and young people ref1
  consciousness ref1
  decoding ref1
  frequencies ref1, ref2, ref3, ref4
  heart ref1
  human energy field ref1
  source, humans as an energy ref1, ref2
  vaccines ref1
  viruses ref1
ennoia ref1
Epstein, Jeffrey ref1, ref2
eternal 'I' ref1, ref2
ethylene oxide ref1
European Union ref1, ref2, ref3, ref4
Event ref1 *and* **Bill Gates** ref2
exosomes, Covid-19 as natural defence mechanism called ref1
experience ref1, ref2
Extinction Rebellion ref1, ref2

F

Facebook
  addiction ref1, 448–50
  Facebook

Archons ref1
censorship ref1, ref2, ref3
hate speech ref1
monopoly, as ref1
private messages, censorship of ref1
Sabbatians ref1
United States election fraud ref1
vaccines ref1
Wetiko factor ref1
fact-checkers ref1
Fauci, Anthony ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10, ref11, ref12
fear ref1, ref2, ref3, ref4
climate change ref1
computer models ref1
conspiracy theories ref1
empty hospitals ref1
Italy ref1, ref2, ref3
lockdowns ref1, ref2, ref3, ref4
masks ref1, ref2
media ref1, ref2
medical staff ref1
Psyop (psychological operation), Covid as a ref1
Wetiko factor ref1, ref2
female infertility ref1
Fermi Paradox ref1
Ferguson, Neil ref1, ref2, ref3, ref4, ref5, ref6, ref7
fertility, decline in ref1
The Field ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
finance *see* **banking, finance and money**
five-senses ref1, ref2
Archons ref1, ref2, ref3

censorship ref1
consciousness, expansion of ref1, ref2, ref3, ref4, ref5, ref6
decoding ref1
education ref1, ref2
the Field ref1, ref2
God, personification of ref1
infinity ref1, ref2
media ref1
paranormal ref1
perceptual programming ref1, ref2
Phantom Self ref1
pneuma not nous, using ref1
reincarnation ref1
self-identity ref1
Wetiko factor ref1, ref2, ref3, ref4, ref5, ref6
5G ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
Floyd, George and protests, killing of ref1
flu, re-labelling of ref1, ref2, ref3
food and water, control of ref1, ref2
Freemasons ref1, ref2, ref3, ref4, ref5, ref6
Frei, Rosemary ref1
frequencies
addictions ref1
Archons ref1, ref2, ref3
awareness ref1
chanting and mantras ref1
consciousness ref1
decoding ref1, ref2
education ref1
electromagnetic (EMF) frequencies ref1
energy ref1, ref2, ref3, ref4
fear ref1

the Field ref1, ref2 5G ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10
five-senses ref1, ref2
ghosts ref1
Gnostics ref1
hive-minds ref1
human, meaning of ref1
light ref1, ref2
love ref1, ref2
magnetism ref1
perception ref1
reality ref1, ref2, ref3
simulation ref1
terror ref1
vaccines ref1
Wetiko ref1, ref2, ref3
Fuellmich, Reiner ref1, ref2, ref3
furlough/rescue payments ref1

G

Gallo, Robert ref1, ref2, ref3
Gates, Bill
Archons ref1, ref2, ref3
climate change ref1, ref2, ref3, ref4
Daily Pass tracking system ref1
Epstein ref1
fascism ref1
five senses ref1
GAVI ref1
Great Reset ref1
GSK ref1
Imperial College ref1, ref2
Johns Hopkins University ref1, ref2, ref3

lockdowns ref1, ref2
masks ref1
Nuremberg trial, proposal for ref1, ref2
Rockefellers ref1, ref2
social distancing and isolation ref1
Sun, dimming the ref1
synthetic meat ref1, ref2
vaccines ref1, ref2, ref3, ref4, ref5, ref6, ref7
Wellcome Trust ref1
Wetiko factor ref1, ref2, ref3
WHO ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10
Wokeness ref1
World Economic Forum ref1, ref2, ref3, ref4
Gates, Melinda ref1, ref2, ref3
GAVI vaccine alliance ref1
genetics, manipulation of ref1, ref2, ref3
Germany ref1, ref2, ref3, ref4, ref5, ref6 *see also* **Nazi Germany**
Global Cult ref1, ref2, ref3, ref4, ref5
anti-human, why Global Cult is ref1
Black Lives Matter (BLM) ref1, ref2, ref3, ref4
China ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9
climate change hoax ref1
contradictory rules ref1
Covid-19 ref1, ref2, ref3
fascism ref1
geographical origins ref1
immigration ref1
Internet ref1
mainstream media ref1, ref2
masks ref1, ref2
monarchy ref1
non-human dimension ref1

perception ref1
political parties ref1, ref2
pyramidal hierarchy ref1, ref2, ref3
reframing ref1
Sabbantian-Frankism ref1, ref2
science, manipulation of ref1
spider and the web ref1
transgender persons ref1
vaccines ref1
who controls the Cult ref1
Wokeness ref1, ref2, ref3, ref4
globalisation ref1, ref2
Gnostics ref1, ref2, ref3, ref4, ref5
Google ref1, ref2, ref3, ref4
government
behavioural scientists and psychologists, advice from ref1, ref2
definition ref1
Joint Biosecurity Centre (JBC) ref1
people, abusive relationship with ref1
Great Reset ref1, ref2, ref3, ref4, ref5, ref6
fascism ref1, ref2, ref3
financial system ref1
Human 2.0 ref1
water and food, control of ref1
green parties ref1
Griesz-Brisson, Margarite ref1
guaranteed income ref1, ref2, ref3

H

Hancock, Matt ref1, ref2, ref3, ref4, ref5
hand sanitisers ref1
heart ref1, ref2

hive-minds/groupthink ref1, ref2, ref3
holographs ref1, ref2, ref3, ref4
hospitals, empty ref1
human, meaning of ref1
Human 2.0 ref1
addiction to technology ref1
artificial intelligence (AI) ref1, ref2
elimination of Human 1.0 ref1
fertility, decline in ref1
Great Reset ref1
implantables ref1
money ref1
mRNA ref1
nanotechnology ref1
parents, replacement of ref1, ref2
Smart Grid, connection to ref1, ref2
synthetic biology ref1, ref2, ref3, ref4
testosterone levels, decrease in ref1
transgender = transhumanism ref1, ref2, ref3
vaccines ref1, ref2, ref3, ref4
human sacrifice ref1, ref2, ref3
Hunger Games Society ref1, ref2, ref3, ref4, ref5, ref6, ref7
Huxley, Aldous ref1, ref2, ref3

I

identity politics ref1, ref2, ref3
Illuminati ref1, ref2
illusory physical reality ref1
immigration ref1, ref2, ref3, ref4
Imperial College ref1, ref2, ref3, ref4, ref5, ref6
implantables ref1, ref2

incomes, destruction of ref1, ref2
Infinite Awareness ref1, ref2, ref3, ref4
Internet ref1, ref2 *see also* social media
artificial intelligence (AI) ref1
independent journalism, lack of ref1
Internet of Bodies (IoB) ref1
Internet of Everything (IoE) ref1, ref2
Internet of Things (IoT) ref1, ref2
lockdowns ref1
Psyop (psychological operation), Covid as a ref1
trolls ref1
intersectionality ref1
inversion
Archons ref1, ref2, ref3
climate change hoax ref1
energy ref1
Judaism ref1, ref2, ref3
symbolism ref1
Wetiko factor ref1
Wokeness ref1, ref2, ref3
Islam
Archons ref1
crypto-Jews ref1
Islamic State ref1, ref2
Jinn and Djinn ref1, ref2, ref3
Ottoman Empire ref1
Wahhabism ref1
isolation *see* **social distancing** *and* **isolation**
Israel
China ref1
Cyber Intelligence Unit Beersheba complex ref1
expansion of illegal settlements ref1

formation ref1
Global Cult ref1
Judaism ref1, ref2, ref3, ref4, ref5
medical experiments, consent for ref1
Mossad ref1, ref2, ref3, ref4
Palestine-Israel conflict ref1, ref2, ref3
parents, replacement of ref1
Sabbatians ref1, ref2, ref3, ref4, ref5
September 11, 2001, terrorist attacks on United States ref1
Silicon Valley ref1
Smart Grid ref1, ref2
United States ref1, ref2
vaccines ref1
Wetiko factor ref1
Italy
fear ref1, ref2, ref3
Lombardy ref1, ref2, ref3
vaccines ref1

J

Johns Hopkins University ref1, ref2, ref3, ref4, ref5, ref6, ref7
Johnson, Boris ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
Joint Biosecurity Centre (JBC) ref1
Judaism
anti-Semitism ref1, ref2, ref3
Archons ref1, ref2
crypto-Jews ref1
inversion ref1, ref2, ref3
Israel ref1, ref2, ref3, ref4, ref5
Labour Party ref1
Nazi Germany ref1, ref2, ref3, ref4
Sabbatians ref1, ref2, ref3, ref4, ref5

Silicon Valley ref1
Torah ref1
United States ref1, ref2
Zionists ref1, ref2, ref3

K

Kaufman, Andrew ref1, ref2, ref3, ref4
knowledge ref1, ref2, ref3, ref4, ref5, ref6
Koch's postulates ref1
Kurzweil, Ray ref1, ref2, ref3, ref4, ref5, ref6, ref7
Kushner, Jared **ref1**, **ref2**

L

Labour Party ref1, ref2
Lanka, Stefan ref1, ref2
Lateral Flow Device (LFD) ref1
Levy, Paul ref1, ref2, ref3
Life Program ref1
lockdowns ref1, ref2, ref3
amplification tampering ref1
Archons ref1
Behavioural Insights Team ref1
Black Lives Matter (BLM) ref1
care homes, deaths in ref1
children
abuse ref1, ref2
mental health ref1
China ref1, ref2
computer models ref1
consequences ref1, ref2
dependency ref1, ref2, ref3

domestic abuse ref1
fall in cases ref1
fear ref1, ref2, ref3, ref4
guaranteed income ref1
Hunger Games Society ref1, ref2, ref3
interaction, destroying ref1
Internet ref1, ref2
overdoses ref1
perception ref1
police-military state ref1, ref2
protests ref1, ref2, ref3, ref4, ref5
psychopathic personality ref1, ref2, ref3
reporting/snitching, encouragement of ref1, ref2
testing ref1
vaccines ref1
Wetiko factor ref1
WHO ref1
love ref1, ref2, ref3
Lucifer ref1, ref2, ref3

M

Madej, Carrie ref1, ref2
Magufuli, John ref1, ref2
mainstream media ref1
BBC ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
censorship ref1, ref2
China ref1
climate change hoax ref1
fear ref1, ref2
Global Cult ref1, ref2
independent journalism, lack of ref1
Ofcom ref1, ref2, ref3

perception ref1, ref2
Psyop (psychological operation), Covid as a ref1
Sabbatians ref1, ref2
social disapproval ref1
social distancing and isolation ref1
United States ref1, ref2
vaccines ref1, ref2, ref3, ref4, ref5
Mao Zedong ref1, ref2, ref3
Marx and Marxism ref1, ref2, ref3, ref4, ref5, ref6
masculinity ref1
masks/face coverings ref1, ref2, ref3
censorship ref1
children ref1, ref2, ref3, ref4, ref5
China, made in ref1
dehumanisation ref1, ref2, ref3
fear ref1, ref2
flu ref1
health professionals ref1, ref2, ref3, ref4
isolation ref1
laughter ref1
mass non-cooperation ref1
microplastics, risk of ref1
mind control ref1
multiple masks ref1
oxygen deficiency ref1, ref2, ref3
police ref1, ref2, ref3, ref4, ref5
pollution, as cause of plastic ref1
Psyop (psychological operation), Covid as a ref1
reframing ref1, ref2
risk assessments, lack of ref1, ref2
self-respect ref1
surgeons ref1

United States ref1
vaccines ref1, ref2, ref3, ref4, ref5
Wetiko factor ref1
'worms' ref1
The Matrix movies ref1, ref2, ref3
measles ref1, ref2
media see mainstream media
Medicines and Healthcare products Regulatory Agency (MHRA) ref1, ref2, ref3, ref4
Mesopotamia ref1
messaging ref1
military-police state ref1, ref2, ref3
mind control ref1, ref2, ref3, ref4, ref5, ref6 *see also* **MKUltra**
MKUltra ref1, ref2, ref3
monarchy ref1
money *see* **banking, finance and money**
Montagnier, Luc ref1, ref2, ref3
Mooney, Bel ref1
Morgellons disease ref1, ref2
mortality rate ref1
Mullis, Kary ref1, ref2, ref3
Musk, Elon ref1

N

Nag Hammadi texts ref1, ref2, ref3
nanotechnology ref1, ref2, ref3
narcissism ref1
Nazi Germany ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
near-death experiences ref1, ref2
Neocons ref1, ref2, ref3

Neuro-Linguistic Programming (NLP) and the Delphi technique ref1
NHS (National Health Service)
amplification cycles ref1
Common Purpose ref1, ref2
mind control ref1
NHS England ref1
saving the NHS ref1, ref2
vaccines ref1, ref2, ref3, ref4, ref5
whistle-blowers ref1, ref2, ref3
No-Problem-Reaction-Solution ref1, ref2, ref3, ref4
non-human dimension of Global Cult ref1
nous ref1
numbers, reality as ref1
Nuremberg Codes ref1, ref2, ref3
Nuremberg-like tribunal, proposal for ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10, ref11, ref12

O

Obama, Barack ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10
O'Brien, Cathy ref1, ref2, ref3, ref4
Ochel, Evita ref1
Ofcom ref1, ref2, ref3
old people ref1, ref2, ref3, ref4, ref5
Oneness ref1, ref2, ref3
Open Society Foundations (Soros) ref1, ref2, ref3
oxygen 406, 528–34

P

paedophilia ref1, ref2
Page, Larry ref1, ref2, ref3, ref4, ref5, ref6, ref7

Palestine-Israel conflict ref1, ref2, ref3
pandemic, definition of ref1
pandemic and health crisis scenarios/simulations ref1, ref2, ref3, ref4
paranormal ref1
PCR tests ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
Pearl Harbor attacks, prior knowledge of ref1
Pelosi, Nancy ref1, ref2, ref3
perception ref1, ref2, ref3, ref4
- climate change hoax ref1
- control ref1, ref2, ref3
- decoding ref1, ref2
- enslavement ref1
- externally-delivered perceptions ref1
- five senses ref1
- human labels ref1
- media ref1, ref2
- political parties ref1, ref2
- Psyop (psychological operation), Covid as a ref1
- sale of perception ref1
- self-identity ref1, ref2
- Wokeness ref1

Phantom Self ref1, ref2, ref3
pharmaceutical industry *see* **Big Pharma**
phthalates ref1
Plato's Allegory of the Cave ref1, ref2
pneuma ref1
police
- Black Lives Matter (BLM) ref1
- brutality ref1
- citizen's arrests ref1, ref2
- common law arrests ref1, ref2

Common Purpose ref1
defunding ref1
lockdowns ref1, ref2
masks ref1, ref2, ref3, ref4
police-military state ref1, ref2, ref3
psychopathic personality ref1, ref2, ref3, ref4
reframing ref1
United States ref1, ref2, ref3, ref4
Wokeness ref1
polio ref1
political correctness ref1, ref2, ref3, ref4
political parties ref1, ref2, ref3, ref4
political puppets ref1
pollution ref1, ref2, ref3
post-mortems/autopsies ref1
Postage Stamp Consensus ref1, ref2
pre-emptive programming ref1
Problem-Reaction-Solution ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
Project for the New American Century ref1, ref2, ref3, ref4
psychopathic personality ref1
Archons ref1
heart energy ref1
lockdowns ref1, ref2, ref3
police ref1, ref2, ref3, ref4
recruitment ref1, ref2
vaccines ref1
wealth ref1
Wetiko ref1, ref2
Psyop (psychological operation), Covid as a ref1, ref2, ref3, ref4, ref5
Pushbackers ref1, ref2, ref3, ref4
pyramid structure ref1, ref2, ref3, ref4

Q

QAnon Psyop ref1, ref2, ref3

R

racism *see also* **Black Lives**
  Matter (BLM)
  anti-racism industry ref1
  class ref1
  critical race theory ref1
  culture ref1
  intersectionality ref1
  reverse racism ref1
white privilege ref1, ref2
white supremacy ref1, ref2, ref3, ref4, ref5
  Wokeness ref1, ref2, ref3
radiation ref1, ref2
randomness, illusion of ref1, ref2, ref3
reality ref1, ref2, ref3
reframing ref1, ref2
  change agents ref1, ref2
  children ref1
  climate change ref1
  Common Purpose leadership programme ref1, ref2
  contradictory rules ref1
  enforcers ref1
  masks ref1, ref2
  NLP and the Delphi technique ref1
  police ref1
  Wetiko factor ref1
  Wokeness ref1, ref2
religion *see also* particular religions
  alien invasions ref1

Archons ref1, ref2
consciousness ref1, ref2
control, system of ref1, ref2, ref3
criticism, prohibition on ref1
five senses ref1
good and evil, war between ref1
hidden non-human forces ref1, ref2
Sabbatians ref1
save me syndrome ref1
Wetiko ref1
Wokeness ref1
repetition and mind control ref1, ref2, ref3
reporting/snitching, encouragement of ref1, ref2
Reptilians/Grey entities ref1
rewiring the mind ref1
Rivers, Thomas Milton ref1, ref2
Rockefeller family ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9
Rockefeller Foundation documents ref1, ref2, ref3, ref4
Roman Empire ref1
Rothschild family ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9
RT-PCR tests ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
Russia
collusion inquiry in US ref1
Russian Revolution ref1, ref2
Sabbatians ref1

S

Sabbantian-Frankism ref1, ref2
anti-Semitism ref1, ref2
banking and finance ref1, ref2, ref3
China ref1, ref2
Israel ref1, ref2, ref3, ref4, ref5

Judaism ref1, ref2, ref3, ref4, ref5
Lucifer ref1
media ref1, ref2
Nazis ref1, ref2
QAnon ref1
Rothschilds ref1, ref2, ref3, ref4, ref5, ref6
Russia ref1
Saudi Arabia ref1
Silicon Valley ref1
Sumer ref1
United States ref1, ref2, ref3
Wetiko factor ref1
Wokeness ref1, ref2, ref3
SAGE (Scientific Advisory Group for Emergencies) ref1, ref2, ref3, ref4
SARS-1 ref1
SARs-CoV-2 ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
Satan/Satanism ref1, ref2, ref3, ref4, ref5, ref6, ref7
satellites in low-orbit ref1
Saudi Arabia ref1
Save Me Syndrome ref1
scapegoating ref1
Schwab, Klaus ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10, ref11, ref12
science, manipulation of ref1
self-identity ref1, ref2, ref3, ref4
self-respect, attacks on ref1
September 11, 2001, terrorist attacks on United States ref1, ref2, ref3, ref4
77th Brigade of UK military ref1, ref2, ref3
Silicon Valley/tech giants ref1, ref2, ref3, ref4, ref5, ref6 *see also* **Facebook**

Israel ref1
Sabbatians ref1
technocracy ref1
Wetiko factor ref1
Wokeness ref1
simulation hypothesis ref1, ref2, ref3, ref4, ref5
Smart Grid ref1, ref2, ref3
artificial intelligence (AI) ref1
China ref1, ref2
control centres ref1
the Field ref1
Great Reset ref1
Human 2.0 ref1, ref2
Israel ref1, ref2
vaccines ref1
Wetiko factor ref1
social disapproval ref1
social distancing *and* **isolation** ref1, ref2, ref3
abusive relationships ref1, ref2
children ref1
flats and apartments ref1
heart issues ref1
hugs ref1
Internet ref1
masks ref1
media ref1
older people ref1, ref2
one-metre (three feet) rule ref1
rewiring the mind ref1
simulation, universe as a ref1
SPI-B ref1
substance abuse ref1

suicide and self-harm ref1, ref2, ref3, ref4, ref5
technology ref1
torture, as ref1, ref2
two-metre (six feet) rule ref1
women ref1
social justice ref1, ref2, ref3, ref4
social media *see also* **Facebook bans on alternative views** ref1
censorship ref1, ref2, ref3, ref4, ref5, ref6
children ref1
emotion ref1
perception ref1
private messages ref1
Twitter ref1, ref2, ref3, ref4, ref5, ref6, ref7
Wetiko factor ref1
YouTube ref1, ref2, ref3, ref4, ref5
Soros, George ref1, ref2, ref3, ref4, ref5, ref6
Spain ref1
SPI-B (Scientific Pandemic Insights Group on Behaviours) ref1, ref2, ref3, ref4
spider and the web ref1, ref2, ref3, ref4
Starmer, Keir ref1
Statute Law ref1
Steiner, Rudolf ref1, ref2, ref3
Stockholm syndrome ref1
streptomycin ref1
suicide and self-harm ref1, ref2, ref3, ref4, ref5
Sumer ref1, ref2
Sunstein, Cass ref1, ref2, ref3
swine flu (H1N1) ref1, ref2, ref3
synchronicity ref1
synthetic biology ref1, ref2, ref3, ref4
synthetic meat ref1, ref2

T

technology *see also* **artificial intelligence (AI); Internet;**
social media addiction ref1, ref2, ref3, ref4
Archons ref1, ref2
the cloud ref1, ref2, ref3, ref4, ref5, ref6, ref7
cyber-operations ref1
cyberwarfare ref1
radiation ref1, ref2
social distancing and isolation ref1
technocracy ref1
Tedros Adhanom Ghebreyesus ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10, ref11, ref12, ref13
telepathy ref1
Tenpenny, Sherri ref1
Tesla, Nikola ref1
testosterone levels, decrease in ref1
testing for Covid-19 ref1, ref2
anal swab tests ref1
cancer ref1
China ref1, ref2, ref3
Corman-Drosten test ref1, ref2, ref3, ref4
death certificates ref1, ref2
fraudulent testing ref1
genetic material, amplification of ref1
Lateral Flow Device (LFD) ref1
PCR tests ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8
vaccines ref1, ref2, ref3
Thunberg, Greta ref1, ref2, ref3
Totalitarian Tiptoe ref1, ref2, ref3, ref4
transgender persons
activism ref1
artificial wombs ref1

censorship ref1
child abuse ref1, ref2
Human 2.0 ref1, ref2, ref3
Wokeness ref1, ref2, ref3, ref4, ref5
women, deletion of rights and status of ref1, ref2
young persons ref1
travel restrictions ref1
Trudeau, Justin ref1, ref2, ref3
Trump, Donald ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10, ref11
Twitter **ref1, ref2, ref3, ref4, ref5, ref6, ref7**

U

UKColumn ref1, ref2
United Nations (UN) ref1, ref2, ref3, ref4, ref5 *see also* **Agenda 21/Agenda 2030 (UN)**
United States ref1, ref2
American Revolution ref1
borders ref1, ref2
Capitol Hill riot ref1, ref2
children ref1
China ref1, ref2
CIA ref1, ref2
Daily Pass tracking system ref1
demographics by immigration, changes in ref1
Democrats ref1, ref2, ref3, ref4, ref5, ref6, ref7
election fraud ref1
far-right domestic terrorists, pushbackers as ref1
Federal Reserve ref1
flu/respiratory diseases statistics ref1
Global Cult ref1, ref2
hand sanitisers, FDA warnings on ref1

immigration, effects of illegal ref1
impeachment ref1
Israel ref1, ref2
Judaism ref1, ref2, ref3
lockdown ref1
masks ref1
mass media ref1, ref2
nursing homes ref1
Pentagon ref1, ref2, ref3, ref4
police ref1, ref2, ref3, ref4
pushbackers ref1
Republicans ref1, ref2
borders ref1, ref2
Democrats ref1, ref2, ref3, ref4, ref5
Russia, inquiry into collusion with ref1
Sabbatians ref1, ref2, ref3
September 11, 2001, terrorist attacks ref1, ref2, ref3, ref4
UFO sightings, release of information on ref1
vaccines ref1
white supremacy ref1, ref2, ref3, ref4
Woke Democrats ref1, ref2

V

vaccines ref1, ref2, ref3
adverse reactions ref1, ref2, ref3, ref4, ref5
Africa ref1
anaphylactic shock ref1, ref2, ref3, ref4
animals ref1, ref2
anti-vax movement ref1, ref2, ref3, ref4, ref5
AstraZeneca/Oxford ref1, ref2, ref3, ref4
autoimmune diseases, rise in ref1, ref2
Big Pharma ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8

bioweapon, as real ref1, ref2
black and ethnic minority communities ref1
blood clots ref1, ref2
Brain Computer Interface (BCI) ref1
care homes, deaths in ref1
censorship ref1, ref2, ref3
chief medical officers and scientific advisers, financial interests of ref1, ref2
children ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10
China ref1, ref2
clinical trials ref1, ref2, ref3, ref4, ref5, ref6
compensation ref1
compulsory vaccinations ref1, ref2, ref3
computer programs ref1
consciousness ref1
cover-ups ref1
creation before Covid ref1
cytokine storm ref1
deaths and illnesses caused by vaccines ref1, ref2, ref3, ref4, ref5
definition ref1
developing countries ref1
digital tattoos ref1
DNA-manipulation ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10
emergency approval ref1, ref2, ref3, ref4, ref5
female infertility ref1
funding ref1
genetic suicide ref1
Global Cult ref1
heart chakras ref1
hesitancy ref1
Human 2.0 ref1, ref2, ref3, ref4
immunity from prosecution ref1, ref2, ref3

implantable technology ref1
Israel ref1
Johnson & Johnson ref1, ref2, ref3, ref4
lockdowns ref1
long-term effects ref1
mainstream media ref1, ref2, ref3, ref4, ref5
masks ref1, ref2, ref3, ref4, ref5
Medicines and Healthcare products Regulatory Agency (MHRA) ref1, ref2
messaging ref1
Moderna ref1, ref2, ref3, ref4, ref5, ref6
mRNA vaccines ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9
nanotechnology ref1, ref2
NHS ref1, ref2, ref3, ref4, ref5
older people ref1, ref2
operating system ref1
passports ref1, ref2, ref3, ref4
Pfizer/BioNTech ref1, ref2, ref3, ref4, ref5, ref6, ref7
polyethylene glycol ref1
pregnant women ref1
psychopathic personality ref1
races, targeting different ref1
reverse transcription ref1
Smart Grid ref1
social distancing ref1
social media ref1
sterility ref1
synthetic material, introduction of ref1
tests ref1, ref2, ref3
travel restrictions ref1
variants ref1, ref2
viruses, existence of ref1
whistle-blowing ref1

WHO ref1, ref2, ref3, ref4
Wokeness ref1
working, vaccine as ref1
young people ref1
Vallance, Patrick ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9
variants ref1, ref2, ref3
vegans ref1
ventilators ref1, ref2
virology ref1, ref2
virtual reality ref1, ref2, ref3
viruses, existence of ref1
visual reality ref1, ref2
vitamin D ref1, ref2
von Braun, Wernher ref1, ref2

W

war-zone hospital myths ref1
waveforms ref1, ref2
wealth ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9 ref10, ref11
wet market conspiracy ref1
Wetiko factor ref1
alcoholism and drug addiction ref1
anti-human, why Global Cult is ref1
Archons ref1, ref2, ref3, ref4
artificial intelligence (AI) ref1
Big Pharma ref1, ref2
children ref1
China ref1
consciousness ref1, ref2
education ref1
Facebook ref1

fear ref1, ref2
frequency ref1, ref2
Gates ref1, ref2
Global Cult ref1, ref2
heart ref1, ref2
lockdowns ref1
masks ref1
Native American concept ref1
psychopathic personality ref1, ref2
reframing/retraining programmes ref1
religion ref1
Silicon Valley ref1
Smart Grid ref1
smartphone addiction ref1, ref2
social media ref1
war ref1, ref2
WHO ref1
Wokeness ref1, ref2, ref3
Yaldabaoth ref1, ref2, ref3, ref4
whistle-blowing ref1, ref2, ref3, ref4, ref5, ref6, ref7
white privilege ref1, ref2
white supremacy ref1, ref2, ref3, ref4, ref5
Whitty, Christopher ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10
'who benefits' ref1
Wi-Fi ref1, ref2, ref3, ref4
Wikipedia ref1, ref2
Wojcicki, Susan ref1, ref2, ref3, ref4, ref5, ref6, ref7
Wokeness
Antifa ref1, ref2, ref3, ref4
anti-Semitism ref1
billionaire social justice warriors ref1, ref2, ref3

Capitol Hill riot ref1, ref2
censorship ref1
Christianity ref1
climate change hoax ref1, ref2
culture ref1
education, control of ref1
emotion ref1
facts ref1
fascism ref1, ref2, ref3
Global Cult ref1, ref2, ref3, ref4
group-think ref1
immigration ref1
indigenous people, solidarity with ref1
inversion ref1, ref2, ref3
left, hijacking the ref1, ref2
Marxism ref1, ref2, ref3
mind control ref1
New Woke ref1
Old Woke ref1
Oneness ref1
perceptual programming ref1
Phantom Self ref1
police ref1
defunding the ref1
reframing ref1
public institutions ref1
Pushbackers ref1, ref2, ref3
racism ref1, ref2, ref3
reframing ref1, ref2
religion, as ref1
Sabbatians ref1, ref2, ref3
Silicon Valley ref1
social justice ref1, ref2, ref3, ref4

transgender ref1, ref2, ref3, ref4, ref5
United States ref1, ref2
  vaccines ref1
  Wetiko factor ref1, ref2, ref3
young people ref1, ref2, ref3
women, deletion of rights and status of ref1, ref2
World Economic Forum (WEF) ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9
World Health Organization (WHO) ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9
  AIDs/HIV ref1
  amplification cycles ref1
  Big Pharma ref1, ref2, ref3
  cooperation in health emergencies ref1
  creation ref1, ref2
  fatality rate ref1
  funding ref1, ref2, ref3
  Gates ref1
  Internet ref1
  lockdown ref1
  vaccines ref1, ref2, ref3, ref4
  Wetiko factor ref1
world number 1 (masses) ref1, ref2
world number 2 ref1
Wuhan ref1, ref2, ref3, ref4, ref5, ref6, ref7 ref8

Y

Yaldabaoth ref1, ref2, ref3, ref4, ref5, ref6
Yeadon, Michael ref1, ref2, ref3, ref4
young people *see also* **children** addiction to technology ref1
  Human 2.0 ref1
  vaccines ref1, ref2

Wokeness ref1, ref2, ref3
YouTube ref1, ref2, ref3, ref4, ref5
WHO 548

Z

Zaks, Tal ref1
Zionism ref1, ref2, ref3
Zuckerberg, Mark ref1, ref2, ref3, ref4, ref5, ref6, ref7, ref8, ref9, ref10, ref11, ref12
Zulus ref1

ICKONIC
THE ALTERNATIVE

Ickonic is something that has been a dream of mine for the last 5 years, growing up around alternative information I have always had a natural interest in what is going on in the World and what could I do to make it better.
Across the range of subjects and positions of influence occupied mainly by people who don't strive to make things better it's the Media that I have always found the most frustrating and fascinating. Mainly because if the Media did their Jobs properly then so much of the negative things happening in the World simply would not be able to happen, because they would be exposed within a heartbeat.
Free Press and the Opportunities that the internet could have given would mean that the Media are able to expose things like never before and hold people to account for their actions. As we all know there are 'Untouchables' that walk among us, people the Media simply won't touch, expose or investigate and that leads to the dark underworlds that infest the establishment the World over.
Well I say enough, it's time for something different, a different kind of Media, where no one is off limits from exposing and investigating. All we're interested in at Ickonic is the truth of what is really going on in the World on whichever subject we're covering.
We hope you enjoy what we have created and take something away from the platform, we aim to deliver information that's informative and most importantly self-empowering, you're not a little person, you're part of something much bigger than that and its time we as a collective race began to understand that and look to the future as ours to take.

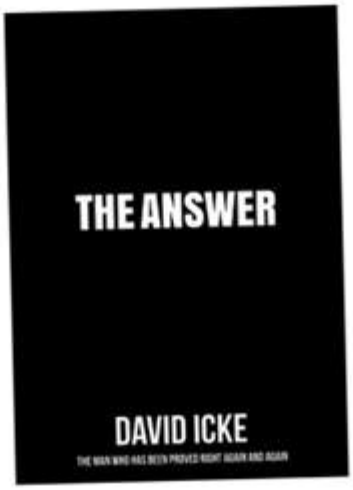
It's time...

Jaymie Icke - Founder Ickonic Alternative Media.

SIGN UP NOW AT ICKONIC.COM

DAVID ICKE

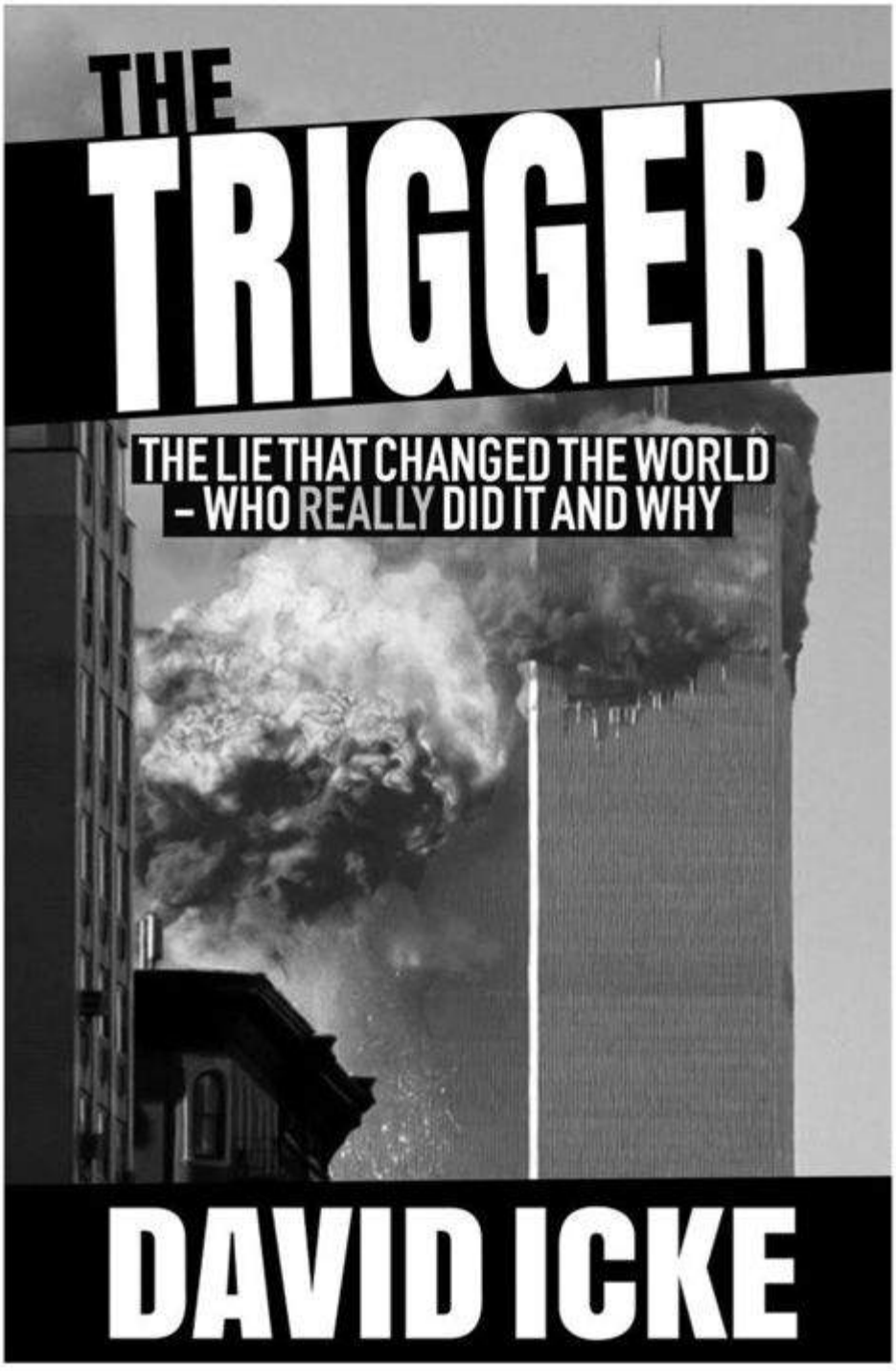
THE ANSWER

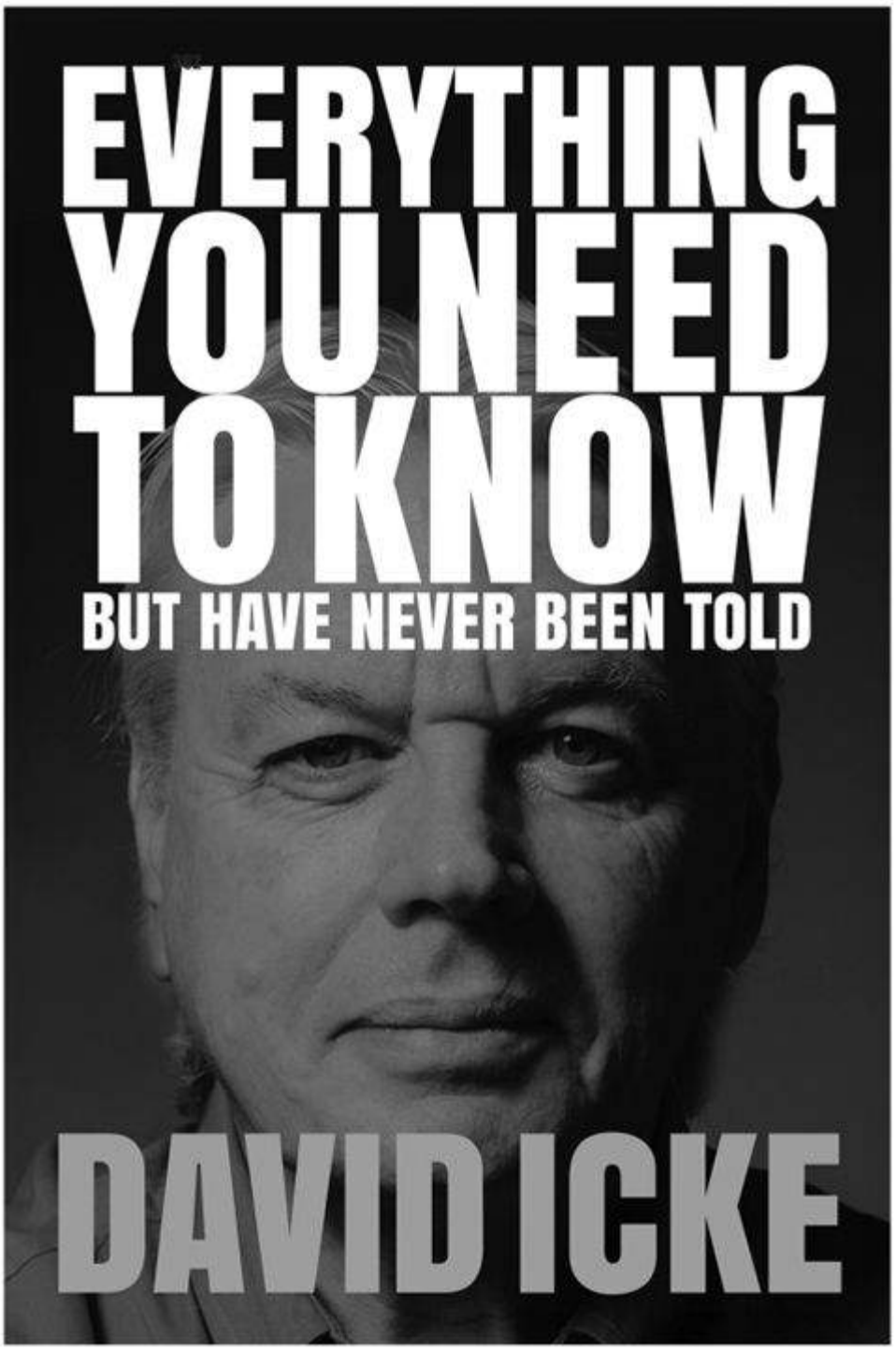


We live in extraordinary times with billions bewildered and seeking answers for what is happening. David Icke, the man who has been proved right again and again, has spent 30 years uncovering the truth behind world affairs and in a stream of previous books he predicted current events.

The Answer will change your every perception of life and the world and set you free of the illusions that control human society. There is nothing more vital for our collective freedom than humanity becoming aware of what is in this book.

Available now at davidicke.com.


THE
TRIGGER
THE LIE THAT CHANGED THE WORLD
– WHO REALLY DID IT AND WHY
DAVID ICKE


EVERYTHING
YOU NEED
TO KNOW
BUT HAVE NEVER BEEN TOLD
DAVID ICKE

DAVIDICKE.COM

DAVID ICKE STORE

LATEST NEWS ARTICLES

DAVID ICKE VIDEOS

WEEKLY DOT-CONNECTOR PODCASTS

LIVE EVENTS

WWW.DAVIDICKE.COM

THE LIFE STORY OF DAVID ICKE

RENEGADE

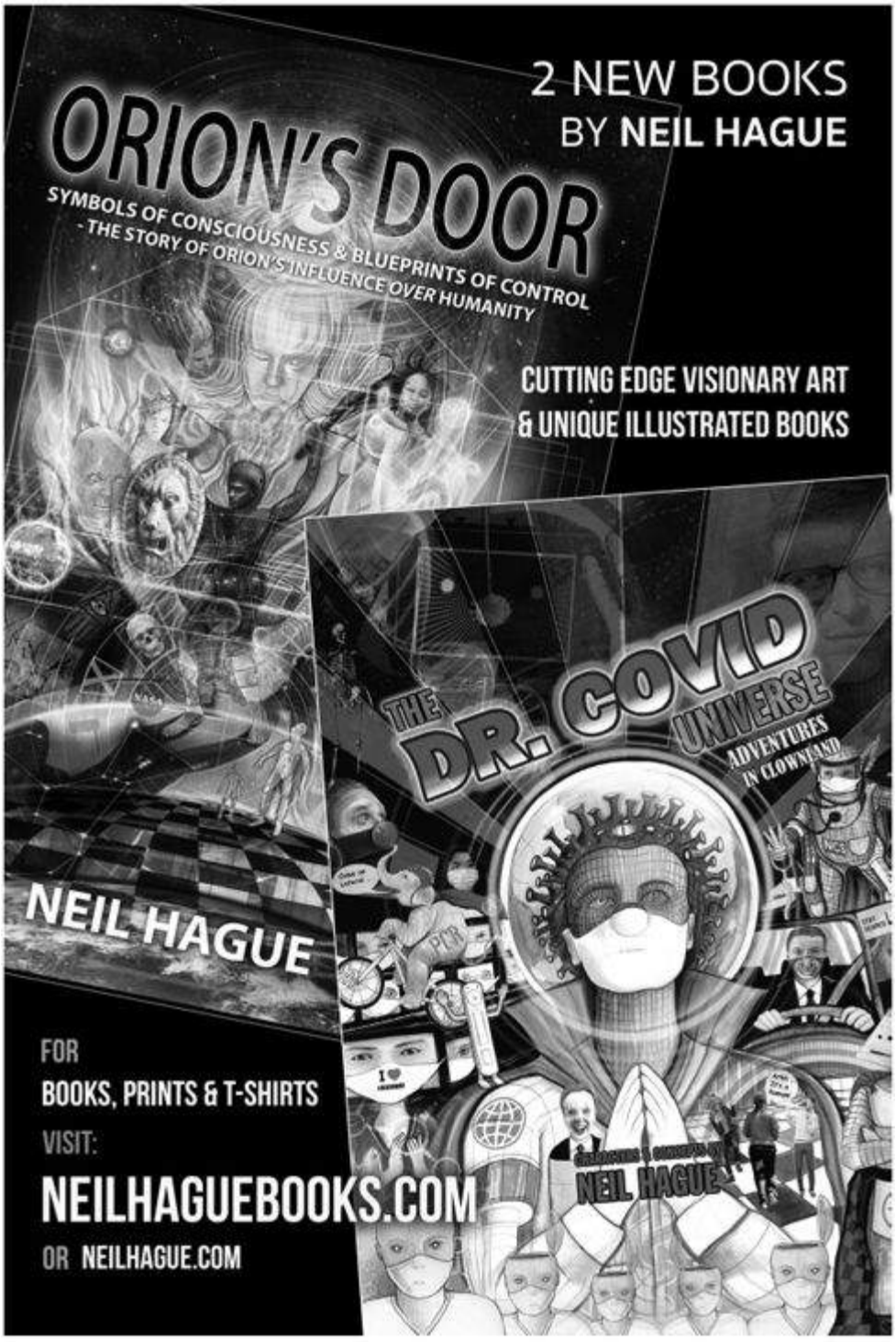
THE FEATURE LENGTH FILM

/ˈren·ɪˌgeɪd/

noun

A person who behaves in a rebelliously unconventional manner.

AVAILABLE NOW AT DAVIDICKE.COM


2 NEW BOOKS
BY NEIL HAGUE
ORION'S DOOR
SYMBOLS OF CONSCIOUSNESS & BLUEPRINTS OF CONTROL
- THE STORY OF ORION'S INFLUENCE OVER HUMANITY
CUTTING EDGE VISIONARY ART
& UNIQUE ILLUSTRATED BOOKS
THE DR. COVID UNIVERSE
ADVENTURES IN CLOWNLAND
NEIL HAGUE
FOR
BOOKS, PRINTS & T-SHIRTS
VISIT:
NEILHAGUEBOOKS.COM
OR NEILHAGUE.COM

Before you go ...

For more detail, background and evidence about the subjects in *Perceptions of a Renegade Mind* – and so much more – see my others books including *And The Truth Shall Set You Free; The Biggest Secret; Children of the Matrix; The David Icke Guide to the Global Conspiracy; Tales from the Time Loop; The Perception Deception; Remember Who You Are; Human Race Get Off Your Knees; Phantom Self; Everything You Need To Know But Have Never Been Told, The Trigger and The Answer*.

You can subscribe to the fantastic new Ickonic media platform where there are many hundreds of hours of cutting-edge information in videos, documentaries and series across a whole range of subjects which are added to every week. This includes my 90 minute breakdown of the week's news every Friday to explain *why* events are happening and to what end.